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Protection by light against heat stress in leaves of tropical crassulacean acid metabolism plants containing high acid levels

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Abstract. Heat tolerance of plants exhibiting crassulacean acid metabolism (CAM) was determined by exposing leaf sections to a range of temperatures both in the dark and the light, followed by measuring chlorophyll *a* fluorescence (F_v/F_m and F_0) and assessing visible tissue damage. Three CAM species, *Clusia rosea* Jacq., *Clusia pratensis* Seem. and *Agave angustifolia* Haw., were studied. In acidified tissues sampled at the end of the night and exposed to elevated temperatures in the dark, the temperature that caused a 50% decline of F_v/F_m (T_{50}), was remarkably low (40−43°C in leaves of *C. rosea*). Conversion of chlorophyll to pheophytin indicated irreversible tissue damage caused by malic acid released from the vacuoles. By contrast, when acidified leaves were illuminated during heat treatments, T_{50} was up to 50−51°C. In de-acidified samples taken at the end of the light period, T_{50} reached ~54°C, irrespective of whether temperature treatments were done in the dark or light. Acclimation of *A. angustifolia* to elevated daytime temperatures resulted in a rise of T_{50} from ~54° to ~57°C. In the field, high tissue temperatures always occur during sun exposure. Measurements of the heat tolerance of CAM plants that use heat treatments of acidified tissue in the dark do not provide relevant information on heat tolerance in an ecological context. However, in the physiological context, such studies may provide important clues on vacuolar properties during the CAM cycle (i.e. on the temperature relationships of malic acid storage and malic acid release).

Additional keywords: *Agave angustifolia*, *Clusia rosea*, *Clusia pratensis*, facultative CAM, obligate CAM, tonoplast.

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Introduction

In their natural habitats, succulent plants exhibiting crassulacean acid metabolism (CAM) (Borland *et al*. 2011) may frequently experience extremely high tissue temperatures during the daytime when stomata are closed and transpirational cooling is essentially nonexistent. Not surprisingly, CAM species adapted to desert climate such as cacti and agaves are among the vascular plants with the highest thermal tolerance levels and heat acclimation potentials (Didden-Zopfy and Nobel 1982; Nobel 1988; Nobel and Zutta 2008). In a field study of nine species of Cactaceae during summertime, the upper thermal tolerance limit determined by chl *a* fluorescence (F_0), was 54.7 ± 2.8°C (Downton *et al*. 1984). According to Nobel (1988), thermal tolerance calculated from the uptake of vital stain neutral red was 62.0 ± 2.1°C in 15 species of *Agave* and 64.0 ± 2.2°C in 18 species of cacti following heat acclimation of plants to 50°C day : 40°C night cycles. Information on the heat tolerance of tropical CAM plants is largely restricted to *Ananas comosus* (L.) Merr., which shows heat tolerance similar to numerous tropical C_3 and C_4 plants (Smillie and Nott 1979; Yamada *et al*. 1996; Weng and Lai 2005). Thermal acclimation that raises the critical temperature threshold has also been found in species performing C_3 photosynthesis, such as annual and perennial desert plants (Downton *et al*. 1984), alpine plants (Braun *et al*. 2002) and temperate rainforest trees (Cunningham and Read 2006). By contrast, leaves of tropical rainforest trees seem to possess little potential to increase the temperature limits of heat tolerance (Cunningham and Read 2006; Krause *et al*. 2010, 2013), although thermal acclimation of physiological processes (e.g. dark respiration and photosynthetic CO_2 assimilation) has been shown to be induced by increased growth temperatures (Krause *et al*. 2013; Slot *et al*. 2014). The low acclimation potential of plants in the humid tropics may be related to plant adaptations to low seasonal temperature changes in their habitats.

Early studies of the CAM plant *Kalanchoë blossfeldiana* Poelln., grown under 12 h light : 12 h dark cycles, demonstrated diel changes in heat tolerance that were interpreted to be related to the CAM cycle (Schwemmle and Lange 1959). Subsequent studies on leaves of greenhouse-grown CAM plants (e.g. of *Aeonium* species) sampled at the end of the dark period when malic acid levels were high, showed very low heat tolerance (Lösch and Kappen 1983; Lehrum *et al*. 1987). In acidified leaves of *Aeonium haworthii* Webb & Berthel., even mild heat stress (39°C) damaged as much as ~20% of the leaf tissue. However, during the course of the light period, as the tissue

acidity gradually declined, heat tolerance increased up to 46.5°C (Kappen and Lösch 1984). The authors suggested that in acidified leaves, malic acid released from the vacuoles under heat stress was damaging the cytoplasmic constituents. A similar relationship between acid content and susceptibility to heat damage was recently shown for the tropical CAM bromeliad *Aechmea blanchetiana* (Baker) L.B.Sm. (Chaves *et al*. 2015). The authors also interpreted their results to be due to cell damage caused by acid release from the vacuoles.

Most importantly, in all these previous studies on CAM plants, although leaves were sampled at different times of the day–night cycle, the actual heat treatments were performed in darkness. In the present investigation, the heat tolerance of three tropical CAM species, *Clusia rosea* Jacq., *Clusia pratensis* Seem., and *Agave angustifolia* Haw., was tested using temperature treatments both in the dark and the light (Krause *et al*. 2010). In addition, we determined the capacity of *A. angustifolia* to upregulate heat tolerance by acclimation to elevated temperatures. The two *Clusia* species are arborescent and inhabit humid, seasonally dry tropical forests. *C. rosea* is a constitutive CAM species (Popp *et al*. 1987) and *C. pratensis* a facultative CAM species (Winter *et al*. 2008; Winter and Holtum 2014). *A. angustifolia*, an obligate CAM species, is native to relatively dry habitats in Central America and Mexico (Winter *et al*. 2014). Experiments were performed during the Panamanian rainy and dry seasons.

Protection by light against heat damage has been observed in isolated spinach (*Spinacia oleracea* L.) chloroplasts (Weis 1982) and leaves of several C_3 species (Schreiber and Berry 1977; Havaux *et al*. 1991), including, more recently, leaves of alpine plants (Buchner *et al*. 2013, 2015) and tropical rainforest trees (Krause *et al*. 2015). Here, we apply heat treatments on acidified and de-acidified CAM leaf tissue both in the light and dark, and show that illumination during heat treatments largely abolishes the high heat sensitivity previously observed in acidified tissues of CAM plants.

Materials and methods

Plant material

Plants were grown under ambient conditions at the Santa Cruz Experimental Research Facility of the Smithsonian Tropical Research Institute in Gamboa (09°07′N, 29°42′W), 30 km from Panama City. Mature leaves of *Clusia rosea* Jacq. and *Clusia pratensis* Seem. (Clusiaceae) were collected from young trees (age: 5–6 years; height: 3–5 m) growing in the ground. *Agave angustifolia* Haw. (Asparagaceae) was cultivated for 2–3 years in forest topsoil in pots 30 cm high and 36 cm wide. Fully developed leaves (length >50 cm) were used. The question whether *A. angustifolia* Haw. is a distinct species or a synonym of *Agave vivipara* L. is discussed in Winter *et al*. (2014).

Three plants of *A. angustifolia* were maintained under increased day temperatures in a fully sunlit glasshouse for ~6 weeks in March 2015. Air temperatures (means ± s.d.; $n = 20$) during 20 days of recording were 44.3 ± 1.7°C (daily maximum) and 24.0 ± 1.5°C (minimum); outside ambient temperatures were 33.1 ± 1.0°C (maximum) and 22.2 ± 1.5°C (minimum). Leaf temperature, recorded at ~1000 hours on fully sun-exposed leaf areas with an infrared thermometer (MiniTemp MT6, Raytek), was 49 ± 3°C ($n = 20$).

Heat tolerance tests

Leaves were harvested either at 0700 hours or at 1600 hours. Heat treatments started ~1 h after harvest. In experiments with *Clusia*, six disks (diameter: 2 cm) per treatment temperature were cut from 6–8 detached leaves. In experiments with *A. angustifolia*, six sections (~4 cm^2) were cut from the central parts (length: ~30 cm) of three *A. angustifolia* leaves. Leaf sections were placed on a wire mesh sheet located 2–3 mm below the water surface of a preheated water bath (Lauda RM6/RMS, Analytical Instruments LLC) and incubated for 20 min at a given temperature in the dark or under ~500 µmol photons m^{-2} s^{-1} PAR, using an 120w Extreme Flower LED (Advanced LED Growth Light). During heating, the abaxial leaf surface was immersed in water, while the adaxial surface remained dry to avoid anaerobiosis (see Krause *et al*. 2015). After heat treatment, leaf sections were stored in Petri dishes at 25−27°C under low light (5–10 µmol photons m^{-2} s^{-1}). Untreated leaf sections served as controls. Chlorophyll *a* fluorescence (F_v/F_m and F_0) was recorded 46–48 h after heat treatments, allowing for recovery of fluorescence parameters. For experiments with *C. rosea* during the dry season (January 2015), fluorescence records performed 8 days after heat treatment are shown (Fig. 1*c*, *d*), as recovery required more than 2 days. Visible tissue damage, seen as light brown colouration, was monitored for up to 8 days. The percentage of damaged leaf area was assessed and averaged (for more details, see Krause *et al*. 2015).

In addition, whole leaves of *C. pratensis* and large leaf sections of *A. angustifolia* were heat-treated in air in the dark using an Isotemp Incubator (Fisher Scientific).

Chlorophyll *a* fluorescence

Leaf sections were dark-adapted for 10 min before recording initial chl *a* fluorescence (F_0) and the ratio of variable to maximum fluorescence (F_v/F_m), an indicator of the potential efficiency of PSII, using a PAM 2000 fluorometer (Walz) as described by Krause *et al*. (2010).

Determination of acid contents

Sections of five different leaves (3–4 cm^2) were stored in liquid N. Organic acids were extracted by boiling in 50% ethanol and reboiling in water. Extracts were titrated with 10 mM KOH to pH 6.5. Means ± s.d. ($n = 5$) of acid contents were recorded.

Detection of pheophytin

Brownish, fully damaged disks (diameter 2 cm) of *C. rosea* leaves harvested in the morning and heat-treated at 53°C in the dark or light, respectively, were incubated for 20 h at 26°C in ethanol (two leaf disks in 10 mL). Absorbance spectra at 600–700 nm of light brown extracts were recorded with a UV–visible light spectrophotometer (UV-2100U, Shimadzu Corporation). The slit width was 2 nm. Pheophytin (Pheo) present in the brownish extract was transformed to the green copper–chlorophyll (Cu-chl) complex according to a method for preparation of chl derivatives as described by Küpper *et al*. (1996). The extract (10 mL) was mixed with an aqueous

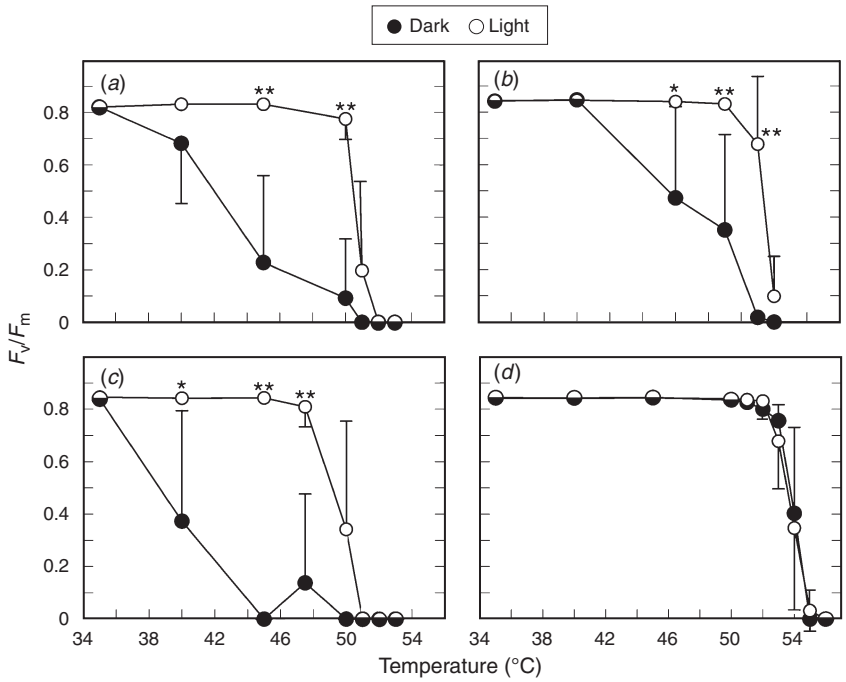

Fig. 1. Response of the ratio of variable to maximum total chl fluorescence, F_v/F_m, to 20 min of heat exposure of leaf disks of *Clusia rosea*. Leaves were harvested at (*a*) 0700 hours and (*b*) 1600 hours in November 2014, and at (*c*) 0700 hours and (*d*) 1600 hours in January 2015. Closed circles, heating in the dark; open circles, heating in the light (~500 µmol photons m^{-2} s^{-1}). Recording of fluorescence was carried out at (*a*, *b*) 46–48 h or (*c*, *d*) 8 days after heat treatment. Means ± s.d. ($n = 6$, sections from different leaves). The F_v/F_m of untreated controls did not differ significantly from the values of leaf sections heated to 35°C. Significant differences in the data between treatment in the dark and light are: **, $P < 0.01$; *, $P < 0.05$. Acid contents: (*a*) 218 ± 71 µmol H^+ g^{-1} FW; (*b*) 248 ± 47 µmol H^+ g^{-1} FW; (*c*) 293 ± 47 µmol H^+ g^{-1} FW; (*d*) 52 ± 17 µmol H^+ g^{-1} FW.

$CuSO_4$ solution to form Cu-chl. For the control, ethanolic extract containing chl (10 mL) was prepared by grinding two untreated leaf disks in the presence of $CaCO_3$ to neutralise organic acids. Chlorophyll was converted to Pheo by the addition of 1 M HCl. The reaction time of Cu-chl formation was <2 min.

Results

Clusia rosea

The heat tolerance of leaves of the obligate CAM species *C. rosea* was tested in the late rainy season, November 2014, and during the early part of the following dry season, January 2015 (Figs 1–4). Leaves with high acid contents of 218 ± 71 µmol H^+ g^{-1} FW, harvested during the rainy season in the morning (Fig. 1*a*), were substantially more sensitive to heat stress in dark than under illumination. The temperatures causing a 50% decline of F_v/F_m (T_{50}), deduced from Fig. 1*a*, were ~43°C for dark-treated and ~51°C for light-treated leaves, respectively. On a very cloudy day, acid content was still high in late afternoon (248 ± 47 µmol H^+ g^{-1} FW), and similar patterns of heat sensitivity of dark- and light-treated leaves harvested at 1600 hours were seen (Fig. 1*b*). The PAR dose of this particular day was low (13.9 mol photons m^{-2} d^{-1}) in comparison to the mean PAR dose in November 2014 of 25.2 ± 7.6 mol photons m^{-2} d^{-1}).

In leaves sampled in the morning during the dry season (acid content 293 ± 47 µmol H^+ g^{-1} FW) (Fig. 1*c*), temperature treatments in the dark resulted in an even higher heat sensitivity (T_{50} ~40°C) compared with treatments in the light (T_{50} ~50°C). At 45–47.5°C in the dark, leaves were almost completely heat-damaged, whereas no or only marginal damage was detected in light-treated tissues. In sharp contrast, de-acidified leaves harvested during the dry season at 1600 hours (Fig. 1*d*) exhibited high heat stability both in dark and light, with T_{50} ~54°C. PAR dose during the study day was 39.3 mol photons m^{-2} d^{-1} and acid content was low (52 ± 17 µmol H^+ g^{-1} FW) when leaves were sampled.

The results of fluorescence measurements for *C. rosea* were corroborated by examination of visible leaf damage. The photograph in Fig. 2 shows heat damage in disks from six individual leaves harvested at 0700 hours during the rainy season. Samples are from the experiment presented in Fig. 1*a*. Visible tissue damage, as demonstrated by light brown colouration, corresponded well with the F_v/F_m values. About 75% of the leaf area was damaged upon exposure to 45°C in the dark, whereas all leaf disks remained undamaged upon exposure to 45°C in the light. At 50°C, close to 90% tissue damage occurred in the dark, but still none in light. The photograph also reveals the typical large variation in heat damage between individual leaves in the critical temperature

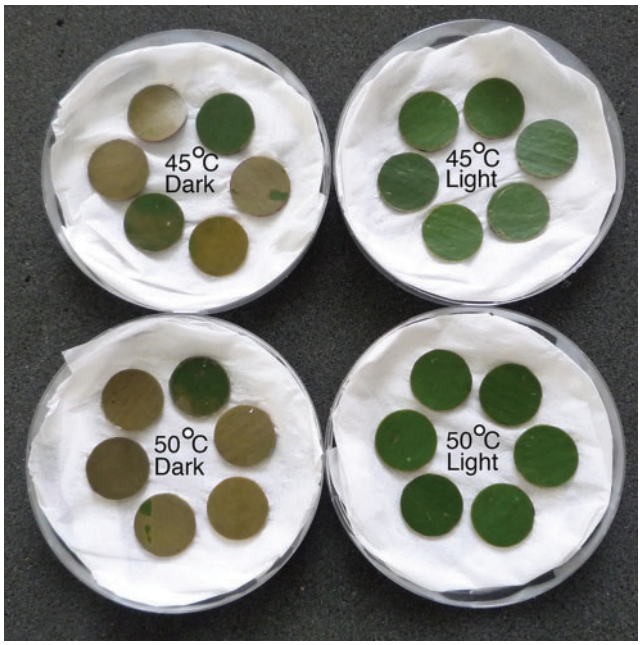

Fig. 2. Images of *Clusia rosea* leaf disks stored subsequent to 20 min of heat treatment for 2 days in dim light. Disks of six different leaves from the experiment of Fig. 1*a* are depicted (leaves harvested at 0700 hours in November 2014). Dishes show samples that were heat-treated at 45°C and 50°C, respectively, in the dark (left) and light (right).

region that resulted in the large s.d. values of mean F_v/F_m data close to T_{50}.

Figure 3 shows the time course of mean visible tissue damage of heat-treated leaf disks of *C. rosea* in experiments during the dry season (see Fig. 1*c*, *d*). In leaves harvested at 0700 hours (Fig. 3*a*), tissue damage reached its final stage after 1 or 2 days. At temperatures close to T_{50}, the brownish colour already appeared during or immediately after heat treatments. Corresponding to the F_v/F_m ratio (Fig. 1*c*), heating in the dark caused 90–100% tissue damage at 47.5°C and 50°C, and ~60% damage at 40°C. In contrast, under illumination, ~60% tissue damage was seen at 50°C and a very low degree of damage (<10%) at 47.5°C.

The mean visible tissue damage shown in Fig. 3*b* corresponds to the fluorescence records of Fig. 1*d*. In leaves with low acid levels, harvested at 1600 hours, damage gradually became visible during the first 4–6 days of storage. Like in the F_v/F_m data, no significant difference between heat effects in the dark and light was seen. After 8 days of storage, ~90% damage was observed upon heating to 55°C, but 53°C and 54°C caused only <20% and ~40% damage, respectively.

The brownish colour of heat-damaged leaf sections resulted from Pheo. Ethanolic extracts of fully damaged leaf sections of *C. rosea* showed a characteristic absorbance band of Pheo *a* in the red spectral region (the Q band) with an absorbance maximum (λ_{max}) at 665 nm (Fig. 4*a*). Upon reaction of an extract containing Pheo with Cu^{2+}, a green solution was obtained and λ_{max} shifted to 652 nm (Fig. 4*b*), characteristic of Cu-chl *a* (cf. White *et al.* 1977). Likewise, the brownish solution obtained by addition of 1 M HCl to extracts of undamaged leaf sections that

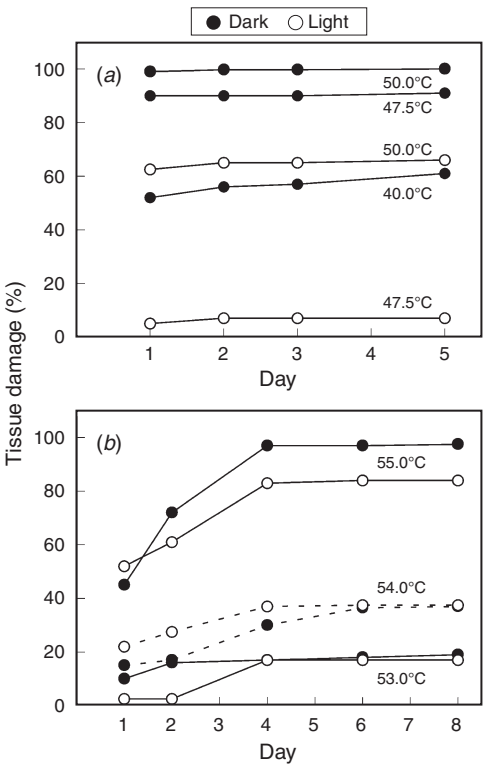

Fig. 3. Visible tissue damage of heat-treated leaf disks of *Clusia rosea* (mean percentage of damaged leaf area of six disks from different leaves) as function of storage time (days). Samples are from the experiments of Fig. 1*c*, *d*: harvest of leaves at (*a*) 0700 hours and (*b*) 1600 hours in January 2015. Heat treatment was in the dark (closed circles) or light (open circles). Treatment temperatures are given in the graph. Above (*a*) 50°C and (*b*) 55°C, all samples exhibited 100% damage; below (*a*) 47.5°C and (*b*) 53°C, all samples remained undamaged.

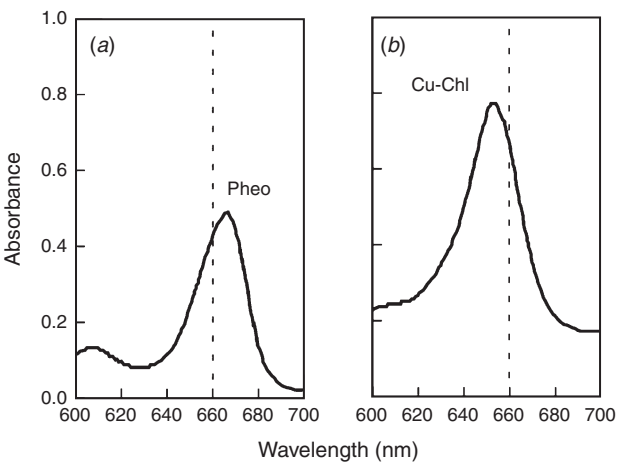

Fig. 4. Absorbance in the red spectral region of ethanolic extracts obtained from fully heat-damaged leaf disks of *Clusia rosea* (see Fig. 1*c*). Leaves were harvested at 0700 hours. (*a*) Spectrum of light-brown extract (pheophytin *a*); λ_{max} of Q-band, 665 nm. (*b*) Spectrum after addition of $CuSO_4$ solution to the extract (green copper–pheophytin *a* complex, Cu-chl *a*); λ_{max} of Q-band, 652 nm.

contained Chl showed that the Q-band of Pheo *a* (λ_{max} = 665 nm) was shifted by reaction with Cu^{2+} to the Q-band of Cu-chl *a* with λ_{max} = 652 nm (data not shown).

Clusia pratensis

In the rainy season (November 2014), leaves of the facultative CAM species *C. pratensis* harvested in the morning did not exhibit significant differences in their response to heat treatment in the dark and light: T_{50} was close to 52°C in both cases (Fig. 5*a*). Corresponding to the F_v/F_m decline, a sharp increase in F_0 was seen between 51°C and 52°C (data not shown). Visible tissue damage developed slowly, reaching ~30% and 60–70% after 6 days upon heating to 52°C and 53°C, respectively (data not shown). In leaves harvested at 1600 hours in the dry season (March 2014; Fig. 5*b*), the course of the decline in F_v/F_m was similar to that in leaves harvested at 0700 hours during the rainy season, although the acid levels were very different (see the legend to Fig. 5 for acid contents). However, in the dry season (February 2015) when high acid levels had accumulated overnight, in leaves harvested at 0700 hours, heat sensitivity was increased in the dark (Fig. 5*c*): T_{50} was 46−47°C in the dark and 50−51°C in the light.

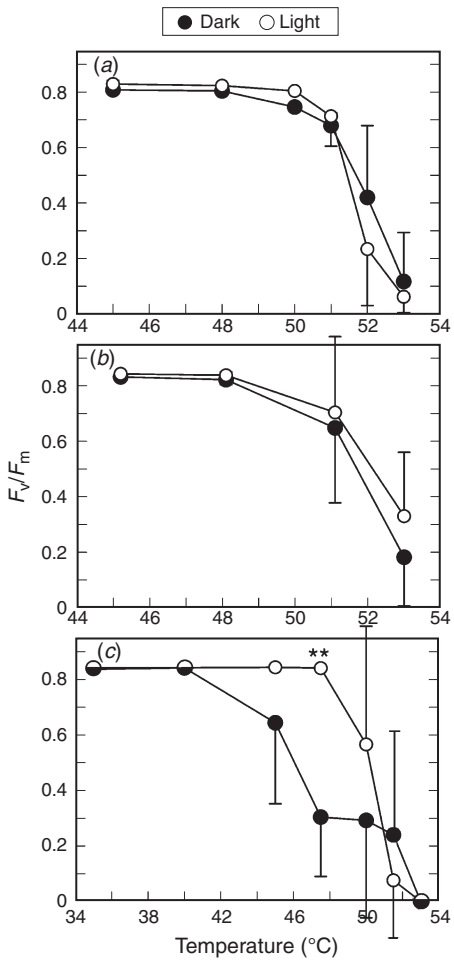

Fig. 5. Response of F_v/F_m to 20 min of heat exposure of leaf disks of *Clusia pratensis*. Leaves were harvested at (*a*) 0700 hours in November 2014, (*b*) at 1600 hours in March 2014 and (*c*) at 0700 hours in February 2015. Closed circles, heating in the dark; open circles, heating in the light. Recording of fluorescence was carried out 46–48 h after heat treatment. Means ± s.d. are given (n = 6, sections from different leaves). The F_v/F_m of untreated controls did not significantly differ from the values of leaf sections heated to 35°C. Significant differences in the data between treatment in dark and light are shown in (*c*): **, $P < 0.01$. Acid contents: (*a*) 131 ± 23 µmol H^+ g^{-1} FW; (*b*) 39 ± 13 µmol H^+ g^{-1} FW; (*c*) 262 ± 79 µmol H^+ g^{-1} FW.

The high heat sensitivity in dark-treated leaf discs of *C. pratensis* harvested at 0700 hours during dry season (see Fig. 5*c*) was confirmed by heating whole leaves in air inside an incubator. Three leaves harvested in the morning and maintained for 30 min in the dark at 48°C showed strong visible damage after 2 days. Brownish areas of leaf blades showed $F_v/F_m = 0$; the remaining green areas had F_v/F_m = 0.658 ± 0.154 (n = 3), indicating slight damage. Three leaves harvested at 1600 hours remained without any visible damage and F_v/F_m was 0.828 ± 0.005 (n = 3). Fig. 6*a* depicts one leaf harvested at 0700 hours (left) and one at 1600 hours (right).

Agave angustifolia

Similar to *C. rosea* and *C. pratensis* (in the dry season), leaves of *A. angustifolia* (grown under ambient conditions) harvested at 0700 hours (acid content 386 ± 9 µmol H^+ g^{-1} FW) were highly heat sensitive in the dark: T_{50} was 43−44°C in the dark and ~51°C in the light (Fig. 7*a*). Leaves harvested at 1600 hours (acid content 13 ± 9 µmol H^+ g^{-1} FW) did not exhibit significant differences in the pattern of F_v/F_m decline between heat treatments in the dark and light (Fig. 7*b*). In both cases, T_{50} was 53−54°C (i.e. higher than in leaves harvested in the morning and heat-exposed in the light).

The high heat sensitivity of acidified *A. angustifolia* leaves in the dark is further demonstrated in Fig. 6*b*. Large leaf sections were incubated in air in the dark at 45°C (left) and 35°C (right). One day after treatment at 45°C, large parts of the leaf area had turned light brown, whereas leaf sections heated to 35°C remained fully green. F_v/F_m ratios recorded 2 days after heat treatment at 45°C were zero in brown areas, whereas F_v/F_m was 0.814 ± 0.010 in the remaining green areas and was not significantly different from that in leaf sections heated to 35°C with F_v/F_m = 0.806 ± 0.007 (n = 3, recorded on different leaf regions).

A. angustifolia grown under increased day temperatures for ~6 weeks (see Materials and methods) acquired a substantially improved heat tolerance. In de-acidified leaves (acid content, 9 ± 5 µmol H^+ g^{-1} FW), heat treatments resulted in T_{50} ~57°C both in the dark and light (Fig. 7*c*) (i.e. T_{50} increased by 3−4°C, compared with plants grown under ambient conditions). The decline in F_v/F_m between 56°C and 58°C correlated closely with a steep rise in F_0 in that temperature range (Fig. 7*d*).

Discussion

The present results obtained for three tropical CAM species under heat treatment in the dark are in agreement with previous heat tolerance studies on various CAM species (Lösch and Kappen 1983; Kappen and Lösch 1984; Lehrum *et al*. 1987), including a tropical bromeliad (Chaves *et al*. 2015). In these studies, as in our investigations, acidified tissue

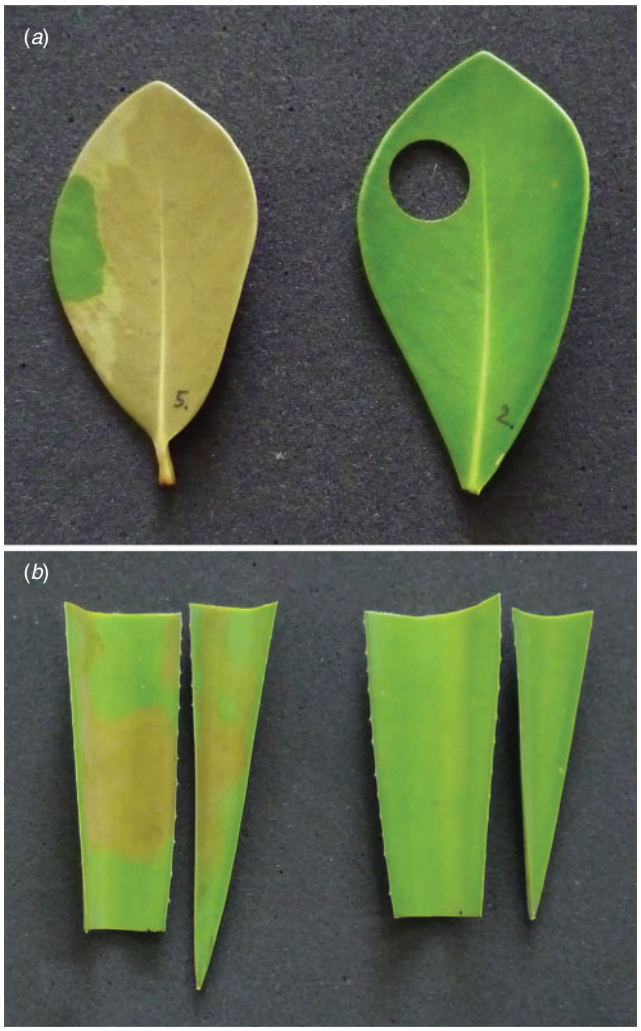

Fig. 6. (*a*) Image of *Clusia pratensis* leaves heated in air for 30 min at 48°C in the dark. Leaves were harvested at 0700 hours (left; cf. Fig. 5*c*) and 1600 hours (right) in February 2015. The photograph was taken 2 days after heat treatment. Length of leaf blades: 8.8 and 10.0 cm for left and right, respectively. (*b*) Image of central and top sections of *Agave angustifolia* harvested at 0700 hours in February 2015 and heated in air for 1 h in the dark at 45°C (left) and 35°C (right). Length of leaf sections: ~15 cm. The photograph was taken 1 day after heat treatment.

sampled at the end of the night and heat-treated in the dark was highly heat-sensitive, whereas the same procedure led to high heat tolerance values in de-acidified tissue harvested in the late afternoon. Here, we demonstrate that heat treatment of acidified tissue in the light largely abolishes the high heat sensitivity reported for acidified tissue, yielding T_{50} values roughly as high as those found in tropical C_3 species (Krause *et al*. 2010, 2015).

Clusia rosea

High heat sensitivity in the dark and effective protection by light in acidified leaf tissue is documented for *C. rosea* in experiments performed during both the rainy and dry seasons (Fig. 1*a–c*). In the de-acidified state, no significant differences in thermal tolerance between heat treatments in the dark and light were observed (Fig. 1*c*): in both the light and dark, T_{50} was raised to ~54°C. The close correlation between F_v/F_m decline and visible tissue damage (Figs 2 and 3) supports the reliability of the chl *a* fluorescence method applied.

In the presence of high acid levels, the characteristic light-brown colouration of damaged leaf tissue, which was observed soon after heat treatment in the dark (Fig. 2), was produced by formation of Pheo from chl. This is evident from the spectra of ethanolic extracts showing the Q-band of Pheo *a* (Fig. 4*a*). Pheo was identified by the formation of the green Cu-chl complex upon addition of $CuSO_4$ (Fig. 4*b*). The wavelengths of absorbance maxima (λ_{max}) in the red spectral region observed here in crude leaf extracts are close to those reported in the literature for purified Pheo *a* and Cu-chl *a* (White *et al*. 1977; Küpper *et al*. 1996; Gerola *et al*. 2011). The slight deviation of our data from earlier publications might be explained by the mixture of chloroplast pigments in the extracts. In Fig. 4, only Q bands are shown, as the other chloroplast pigments (i.e. carotenoids), absorb light in the blue but not in the red spectral region. For chl *a* in acidic ethanolic medium, a 'demetalation' pH of 3.5 (denoting replacement of Mg^{2+} with two protons in the phorphyrin ring system, forming Pheo) was determined by Gerola *et al*. (2011). In contrast, Cu-chl *a* has been reported to be highly stable in acidic conditions.

The results indicate that malic acid and possibly other acids accumulated overnight are released from vacuoles already under moderate heat stress, leading to severe leaf damage in the dark. Noticeably, in a study of the obligate CAM cultivar *Aechmea* 'Maya' (a cross between *A. tessmannii* Harms and *A. fasciata* (Lindl.) Baker) (Ceusters *et al*. 2011), plants transferred to deep shade at dawn exhibited extended brown spots on young fully developed leaves within 8 h, independent of heat stress. The authors attributed this cell damage to overacidification of the cytoplasm.

Enhanced efflux of malic acid across the tonoplast, induced by elevated temperatures, supposedly results from increased membrane fluidity (Kluge and Schomburg 1996). Heat acclimation of CAM plants has been reported to be accompanied by decreased tonoplast fluidity (Kliemchen *et al*. 1993). At high vacuolar acid concentrations, efflux is thought to occur by passive diffusion of undissociated malic acid (H_2mal) (Lüttge and Smith 1984), whereas at lower acid levels, probably the dissociated forms ($Hmal^-$ and mal^{2-}) are transferred across the tonoplast together with stoichiometric H^+ cotransport (see also Smith *et al*. 1996).

The data presented here suggest that the accumulated acid, released upon heat stress in the dark, penetrates into the chloroplasts and converts chl to Pheo. However, when photosynthetic tissue is subjected to heat stress under photosynthetically active light, acid released from the vacuoles is likely to be metabolised, minimising or preventing damage. Additional protective light effects may include formation of zeaxanthin via the xanthophyll cycle. Slight amelioration of heat tolerance by light has been observed also in leaves of tropical C_3 species (Krause *et al*. 2015).

Even under illumination, the heat tolerance of acidified *C. rosea* leaves was limited to a T_{50} of ~50−51°C, which is considerably lower than what was observed in de-acidified

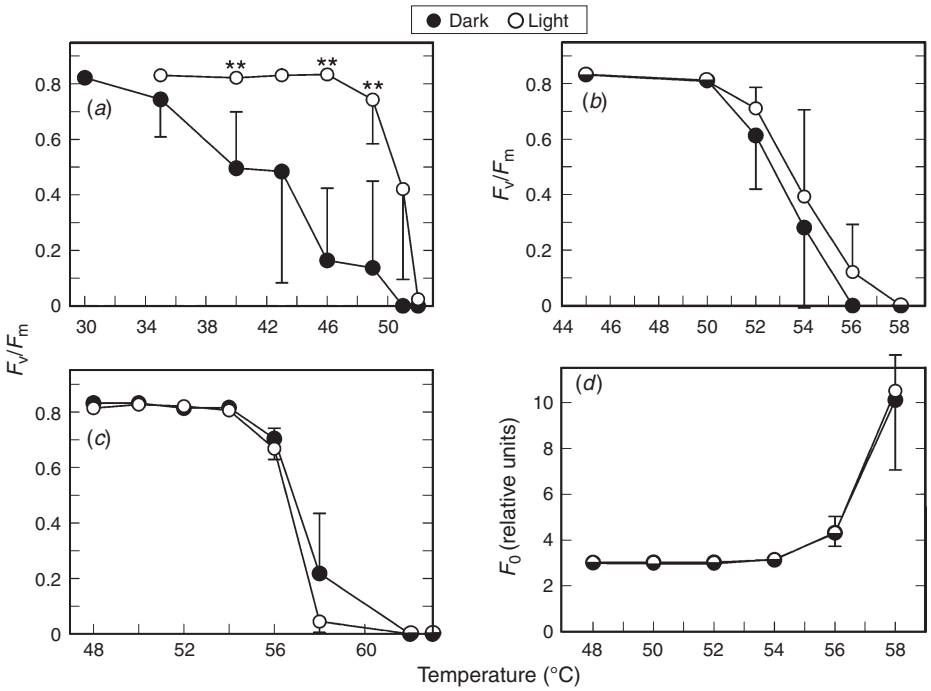

Fig. 7. Response of chl fluorescence parameters to 20 min of heat exposure in the dark (closed symbols) and light (open symbols) of leaf sections of *Agave angustifolia* grown under (*a*, *b*) ambient conditions or (*c*, *d*) acclimated to elevated day temperatures. Note the differences in temperature scales. Recording of fluorescence was carried out 46–48 h after heat treatment. Means ± s.d. ($n = 6$, sections from different leaves). The F_v/F_m ratio of leaves harvested (*a*) at 0700 hours in March 2015 and (*b*) at 1600 hours in Februaruy 2015. The F_v/F_m of untreated controls was (*a*) 0.821 ± 0.014 and (*b*) 0.836 ± 0003. Acid contents: (*a*) 386 ± 9 µmol H^+ g^{-1} FW; (*b*) 13 ± 3 µmol H^+ g^{-1} FW. Significant differences in the data between treatments in the dark and light are shown in (*a*): **, $P < 0.01$; *, $P < 0.05$. (*c*) The F_v/F_m ratio and (*d*) initial fluorescence, F_0 of leaves of high-temperature acclimated plants harvested at 1700 hours in March 2015. The F_v/F_m and F_0 of the controls did not significantly differ from the data of leaf sections heated to 48°C. Acid content: 9 ± 5 µmol H^+ g^{-1} FW.

leaves (T_{50} ~54°C). A similar result was observed in leaves of *A. angustifolia* (see below). In the temperature range above 50°C, malic acid released from the vacuoles might not be turned over fast enough by CAM- and C_3- specific metabolic reactions to avoid damage.

Clusia pratensis

Clusia pratensis belongs to the group of facultative CAM species characterised by the optional use of CAM under drought conditions (Winter and Holtum 2014; Winter *et al.* 2015). Accordingly, in the rainy season, *C. pratensis* was in C_3 mode. Despite elevated acid levels during the entire diel cycle (see Holtum *et al.* 2004), leaves with an acid content of 131 ± 23 µmol H^+ g^{-1} FW, harvested at 0700 hours, did not show significant differences in heat tolerance when heat treatments were performed in dark and light (Fig. 5*a*), in contrast to leaves of *C. rosea* (cf. Fig. 1*a*) containing high acid levels (218 ± 71 µmol H^+ g^{-1} FW). In the late dry season (February–March), when *C. pratensis* was in the CAM mode, leaves with very low acid content (harvested at 1600 hours) exhibited a similar F_v/F_m decline to that seen in the rainy season (i.e. there was no significant difference between treatments in the dark and light) (Fig. 5*b*). When harvested at 0700 hours (262 ± 79 µmol H^+ g^{-1} FW), the leaves showed highly reduced T_{50} values upon heat treatment in the dark (Fig. 5*c*), as in *C. rosea*. Again, the characteristic light-brown colour of heat-damaged leaf tissue (Fig. 6*a*) confirmed the conversion of chl to Pheo by acid released from the vacuoles.

Agave angustifolia

Experiments with plants of the constitutive CAM species *A. angustifolia* grown under ambient conditions corroborated the results for *C. rosea* and *C. pratensis*. Responses of F_v/F_m ratios to heat stress were similar as in *C. rosea* and *C. pratensis* (when in CAM mode) (Fig. 7*a*, *b*). In leaf sections containing high acid levels that were heat-treated in air (Fig. 6*b*), as well as in the water bath (data not shown), the brownish coloration of damaged tissue indicated the formation of Pheo.

In contrast to seedlings of tropical C_3 tree species, which did not show significant increases in heat tolerance following growth at elevated temperatures (see Introduction), considerable heat acclimation potential was demonstrated for *A. angustifolia* by long-term plant exposure to increased daytime temperatures (daily maxima ~11°C above ambient). In de-acidified tissue, the heat tolerance limit rose from 54°C to 57°C in acclimated plants, as indicated by a sharp decline in F_v/F_m and increase in F_0 between 56°C and 58°C (Fig. 7*c*, *d*). These data are comparable to the tolerance limit of *Agave americana*

L. (55.9°C) determined by Downton *et al*. (1984) under summer field conditions near Organ Pipe National Monument, Arizona.

Conclusion

The unusually high heat sensitivity of acidified leaves of CAM plants, assayed in the dark, is probably related to the uncontrolled heat-induced release of malic acid from the vacuoles. Illumination during heat treatments largely eliminates this sensitivity. When not being turned over in an orderly metabolism in photosynthetically active light, malic acid may lead to fatal pH decreases in cytosol and cell organelles, as shown by the transformation of chlorophyll to Pheo. In the field, elevated tissue temperatures typically occur during periods of high irradiance. Therefore, heat treatments performed in the dark on leaves that store high levels of malic acid do not provide a realistic picture of heat tolerance in CAM plants. Nonetheless, from a physiological point of view, specifically designed high-temperature studies with acidified CAM leaves may be informative about the vacuolar properties for malic acid storage and efflux, or, when combined with studies of CO_2 exchange, about the control of malate decarboxylation. There have been reports of substantial CO_2 efflux from leaves of CAM plants at high tissue temperatures during the daytime *in situ* (Cernusak *et al*. 2008), probably related to an imbalance between the release of malic acid from the vacuole and its decarboxylation, the gluconeogenic processing of C_3 products and refixation of CO_2 by Rubisco. Our results show that even under illumination, acidified leaves did not fully reach the high T_{50} values observed with de-acidified leaves. Thus in intact leaves experiencing elevated temperatures and PAR *in situ*, adverse effects of malic acid on photosynthetic metabolism during the de-acidification phase cannot be excluded.

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