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Interactive effects of high irradiance and moderate heat on photosynthesis, pigments, and tocopherol in the tree-fern *Dicksonia antarctica*

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Abstract. Effects of high irradiance and moderate heat on photosynthesis of the tree-fern *Dicksonia antarctica* (Labill., Dicksoniaceae) were examined in a climate chamber under two contrasting irradiance regimes (900 and 170 μmol photons m^{-2} s^{-1}) and three sequential temperature treatments (15°C; 35°C; back to 15°C). High irradiance led to decline in predawn quantum yield of photochemistry, F_v/F_m (0.73), maximal Rubisco activity (V_{cmax}; from 37 to 29 μmol $m^{-2}s^{-1}$), and electron transport capacity (J_{max}; from 115 to 67 μmol m^{-2} s^{-1}). Temperature increase to 35°C resulted in further decreases in F_v/F_m (0.45) and in chlorophyll bleaching of high irradiance plants, while V_{cmax} and J_{max} were not affected. Critical temperature for thylakoid stability (T_c) of *D. antarctica* was comparable with other higher plants (*c.* 47°C), and increases of T_c with air temperature were greater in high irradiance plants. Increased T_c was not associated with accumulation of osmotica or zeaxanthin formation. High irradiance increased the xanthophyll cycle pigment pool (V+A+Z, 91 *v.* 48 mmol mol^{-1} $chlorophyll^{-1}$), de-epoxidation state (56% *v.* 4%), and α-tocopherol. Temperature increase to 35°C had no effect on V+A+Z and de-epoxidation state in both light regimes, while lutein, β-carotene and α-tocopherols increased, potentially contributing to increased membrane stability under high irradiance.

Additional keywords: α-tocopherols, β-carotene, critical temperature, de-epoxidation state, J_{max}, light regime, lutein, osmolality, photoinhibition, V_{cmax}, temperature, xanthophyll pigments.

Introduction

While understorey species of evergreen forests often experience high intensity sunflecks, they are not usually exposed to prolonged periods of high irradiance (Lovelock *et al*. 1998; Tausz *et al*. 2005). A protective canopy usually creates a favourable microclimate with more moderate temperature fluctuations and greater air humidity than in the above-canopy atmosphere. However, during the course of forest ecosystem dynamics including gap formation, bushfires, or anthropogenic management such as forest harvesting, understorey species may be suddenly exposed to full sunlight and high temperatures, stress factors that can contribute to temporary decline of these species. According to climate-change projections, these factors are likely to become even more significant in the future, as temperatures are predicted to increase, and disturbances in forest canopies may become more frequent (Hennessy *et al*. 2007).

In the short-term, exposure of shade-acclimated plants to high levels of irradiance often leads to photoinhibition and photo-oxidative stress. Photoinhibition alone is rarely responsible for plant mortality and the plant may recover and become fully acclimated. Photo-oxidative stress is caused by the toxic effects of reactive oxygen species (ROS) produced in the photosynthetic apparatus under high irradiance when carbon assimilation is light-saturated (Niyogi 2000). Many plants can, to a certain extent, acclimate to increased irradiance through enhanced dissipation of absorbed light energy in the thylakoids, a process related to the conversion of the light harvesting xanthophyll violaxanthin to the energy quenching zeaxanthin (Demmig-Adams and Adams 2006). Protection against high irradiance can also involve the accumulation of tocopherol (Munné-Bosch 2005), an antioxidant that scavenges toxic ROS and contributes to thylakoid membrane stability.

When, in addition to high irradiance, leaves are exposed to other environmental stress factors such as high temperature, there can be sustained reductions in the efficiency of photosynthetic energy conversion and inhibition of repairs to photodamaged photosystem II (PSII; Murata *et al*. 2007). Photosynthesis is particularly sensitive to inhibition by heat stress due to labile components in the photosynthetic apparatus (Salvucci and Crafts-Brandner 2004). The thylakoid membrane is one of the main temperature stress targets and changes during acclimation occur at that level (Ducruet *et al*. 2007). The degree of thermostability of the thylakoids can be estimated by the critical temperature T_c – the temperature threshold above which irreversible damage occurs to PSII (Schreiber and Berry 1977). T_c changes with growing conditions, reflecting thermal acclimation of the

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photosynthetic apparatus (Ducruet *et al*. 2007). Chlorophyll fluorescence yield is a sensitive indicator of the state of thylakoids, and can be used to assess T_c in plants as the temperature threshold above which ground level fluorescence (F_o) increases (e.g. Froux *et al*. 2004). The mechanisms underlying acclimatory changes in T_c are still poorly understood, although some results point towards stabilising effects of protective compounds on thylakoids. For example, the xanthophyll zeaxanthin (Havaux and Gruszecki 1993; Havaux and Tardy 1996), as well as increased soluble sugar concentration (Hüve *et al*. 2006), are believed to have a stabilising effect and shift T_c towards higher temperatures.

Our model understorey species, the tree-fern *Dicksonia antarctica* (Labill., Dicksoniaceae), is known to decline after clear-fell logging in Victoria, Australia (Ough and Murphy 2004). These tree-ferns are iconic and ecologically significant understorey species in many humid forest types in the southern hemisphere, including Australian temperate rain forests and wet sclerophyll (eucalypt) forests (Large and Braggins 2004). They support a large epiphytic diversity on their trunks and provide nursery sites for many tree and shrub species as well as nesting and feeding sites for marsupials, insects and birds (Magrath and Lill 1983; Lindenmayer *et al*. 1994; Roberts *et al*. 2005). Decline in *D. antarctica* numbers is expected to negatively impact on many dependent species and thus maintenance of tree-ferns is often an objective of forest management plans (Department of Natural Resources and Environment 2002).

The reasons for poor survival and ongoing decline of *D. antarctica* after logging remain uncertain (Ough and Murphy 2004), but exposure to high irradiance, combined with increased air and frond temperatures, could be contributing factors. Periodically disturbed by wildfires in their natural habitat, *D. antarctica* are exposed to a broad range of irradiance during their lifetime (Hunt *et al*. 2002), which suggests that this species is able to at least partly acclimate to different levels of irradiance. Certainly, other studies indicate potential for fern acclimation to different light regimes. For example, New Zealand ferns from contrasting habitats displayed contrasting characteristics in terms of photosynthetic light compensation point, which were tightly correlated with specific frond area (Bannister and Wildish 1982). Frond characteristics (frond surface area, epidermis thickness, palisade/spongy mesophyll ratio, blade size, petiole length) of a South American *Cyathea* species (another important tree fern genus) were also correlated with its local irradiance (Arens 1997). However, other studies suggest limited capacity of shade-acclimated tree-ferns to efficiently adjust to increased irradiance (Durand and Goldstein 2001). To our knowledge, only a few studies have examined effects of high temperature, either alone or with high irradiance on the physiological performance of tree-ferns: Tingey *et al*. (1987) found that photosynthesis of *D. antarctica* was particularly susceptible to inhibition with increasing temperature and high light; and Nobel *et al*. (1984) also mentioned negative effects of high temperature on gas exchange of ferns. Natural distribution of *D. antarctica* in Australia is limited to the temperate zone (McCarthy 1998), characterised by cool to warm conditions (Köppen classification; Australian Bureau of Meteorology, http://www.bom.gov.au/iwk/climate_zones, verified 13 August 2009), perhaps indicating that the species has limited potential for acclimation to temperature increases (such as after clear-fell logging, but potentially also due to climate change), making it susceptible to ongoing decline.

In this study, we investigated the responses of *D. antarctica* to high irradiance, moderately high temperature (+35°C), and a combination of both under fully controlled climate chamber conditions. Measuring T_c together with several variables related to photosynthesis, chlorophyll fluorescence, and chloroplast pigments, we addressed the following specific questions:

(1) Are photosynthetic parameters of *D. antarctica* adversely affected by (a) high irradiance; (b) high temperatures; and (c) their interactions?
(2) Does membrane stability (measured via critical temperature, T_c) increase in *D. antarctica* fronds with increased temperature (indicative of an acclimation to high temperature), and if yes, are T_c changes associated with accumulation of osmotica or zeaxanthin formation?
(3) Do other potentially protective thylakoid compounds, such as carotenoids and tocopherol, change in relation to high irradiance and high temperature?
(4) Are effects of high temperature on the above parameters reversible?

Materials and methods

Plant material

One-year-old sporophytes of *Dicksonia antarctica* Labill (HSK Gardening and Leisure Avon Dassett, UK) were transplanted into 10-L pots, containing a mixture of sand and peat (50/50, v/v) and 40 g slow release fertiliser (Nutricote 100, Chisso-Asahi Fertilizer Co. Ltd, Tokyo, Japan; N/P/K, %, 13/13/13) per pot. The plants were grown under uniform sunlit conditions in a naturally illuminated glasshouse at INRA, Champenoux, France (48°44′N, 6°14′E) for 2 months in spring 2007. At the end of this period, the plants were transferred to a climate chamber (Chambre Phytotronique STRADER, Angers, France) at Champenoux.

Climate chamber conditions and experimental design

Irradiance in the climate chamber was provided by two types of 400 W lamps (HQI Philips (mercury halide) and SONT Philips (sodium halide), Koninklijke Philips Electronics N.V., Eindhoven, The Netherlands) and resulted in a photosynthetic photon flux density (PPFD) of 900 μmol photons m^{-2} s^{-1} at plant height (PAR range, 400–700 nm, measured with a Li-Cor quantum sensor; Li-Cor, Lincoln, Nebraska, USA). Relative humidity was 70–80%, air temperature was controlled to ±0.5°C (see temperature treatments below), and photoperiod was 16 h day^{-1}.

The tree-ferns were randomly assigned to two experimental groups ($n = 7$ in each). One group was shielded by a wavelength-neutral shade mesh (17% light transmission, PPFD: 170 μmol photons m^{-2} s^{-1}) – 'shade', the other exposed to full light (900 μmol photons m^{-2} s^{-1}) – 'high irradiance'. Such levels are representative of irradiance conditions in the open on overcast winter and clear summer days respectively, at typical *D. antarctica* field sites in mountain-ash forests of Victoria,

Australia. Plants were kept well watered at all times and were rotated daily at random within their designated irradiance regime.

A sequence of temperature treatments was applied as follows:

(1) 10 days at 15°C day and night;

(1a) 3 days at 25°C day and night;

(2) 12 days at 35°C/25°C day/night (typical hot summer days for *D. antarctica* in the field);

(3) 10 days at 15°C day and night (to check reversibility of temperature effects).

Chlorophyll *a* fluorescence and critical frond temperature were measured for each individual every 1 to 2 days throughout the experiment. Photosynthesis was recorded from net CO_2 uptake (A) *v.* intercellular CO_2 concentration (A-C_i curves) on the first day of the experiment and at the end of each temperature treatment. Samples for nitrogen (N) content, xanthophyll analyses, and osmolality were taken at the end of each of the three temperature treatments. All measurements were made on the mid-third of the youngest fully-expanded fronds that were of healthy appearance (i.e. not discoloured).

Total chlorophyll content was measured for each individual every 1 to 2 days throughout the experiment; measurements were made over the entire plant irrespective of frond condition.

Frond temperature (T_{frond})

Frond temperature (as 10 random points across the entire plant) was measured twice during the 35°C temperature treatment (beginning/end) at predawn and midday, and once at the end of the experiment (during the second 15°C temperature treatment), using an IR laser thermometer (Raynger PM, Raytech Inc., Santa Cruz, CA, USA).

Maximal quantum yield of photochemistry (F_v/F_m)

F_v/F_m was derived from chlorophyll *a* fluorescence measured on dark-acclimated fronds (at the end of the 'night' period) with a modulated fluorometer (PAM 2000, Heinz Walz GmbH, Effeltrich, Germany). Maximum quantum yield of PSII was estimated as $F_v/F_m = (F_m - F_o)/F_m$, after Maxwell and Johnson (2000).

Gas exchange measurements

Gas exchange was measured with a Li-Cor 6400 portable photosynthesis measurement device, equipped with a 2 × 3 cm broadleaf chamber with red-blue LEDs (Li-Cor). All gas exchange measurements were conducted at the reference frond temperature of 25°C. For each plant, an A-C_i curve was generated at PPFD 1000 µmol m^{-2}s^{-1}, frond temperature 25°C, air flow rate 400 µmol air s^{-1}, and relative humidity (RH) >60%. Photosynthesis was induced at a CO_2 mole fraction of 50 µmol mol^{-1} for 15–20 min before measurements to ensure maximal stomatal opening and maximal activity of Calvin cycle enzymes. CO_2 mole fraction was then increased in 13 successive steps to 2200 µmol mol^{-1} with two measurements at each step. After finishing the A-C_i curve, illumination in the leaf chamber was turned off, CO_2 mole fraction was decreased to 400 µmol mol^{-1} and respiration rate was recorded after 5 min in the dark. The frond area enclosed in the Li-Cor chamber was marked, photographed and calculated using imaging software (UTHSCSA Image Tool Version 3, University of Texas, USA). Values for A_{max} and g_s at ambient CO_2 (400 µmol mol^{-1}) were derived from these curves.

Using a biochemical photosynthetic model (Farquhar *et al.* 1980), apparent (i.e. assuming that mesophyll conductance to CO_2, g_i, is infinite) maximum carboxylation rate (apparent V_{cmax}^{25}), and the maximum apparent rate of electron transport (apparent J_{max}^{25}) were estimated by fitting the A-C_i curves to the model as described in Montpied *et al.* (2009). Triose phosphate use (TPU) limitation was not included in the model; points with decreasing A at high CO_2 mole fractions were disregarded. A set of primary parameters of Rubisco kinetic properties used herein, $K_c = 327$ µmol mol^{-1}, $K_o = 282\,600$ µmol mol^{-1}, $\Gamma^* = 43.7$ µmol mol^{-1}, were taken from von Caemmerer *et al.* (1994).

Values of g_i were then estimated with the curve fitting approach introduced by Ethier and Livingston (2004) and described by Montpied *et al.* (2009) and real V_{cmax}^{25} and J_{max}^{25} were computed based on the A-C_c (chloroplastic CO_2 mole fraction); therefore, only corrected V_{cmax} and J_{max} (i.e. under the hypothesis of finite g_i) are given and discussed in this study.

Frond nitrogen and chlorophyll content

Frond samples were analysed for nitrogen (N) content using an elemental analyser (NCS 2500, CE instrument Thermo Quest, Milano, Italy). Samples were dried at 60°C for 48 h (to determine dry weight) and then ground to a fine powder. Frond area of fresh samples was scanned and calculated using Scion Image software (Scion Corporation 2000–2001, USA), and these data used to calculate N content on a frond area basis.

Frond chlorophyll content was estimated from transmittance values measured with the Minolta SPAD-502 chlorophyll meter (Minolta, Illinois, USA; hereafter 'SPAD'). Values were the mean of two to three separate pinnules per plant (randomly selected irrespective of frond colour).

Total chlorophyll ($a + b$) was also measured by HPLC (see Pigments and tocopherol determination).

Critical temperature (T_c)

Critical temperature was estimated *in vivo* from the sharp rise of basal chlorophyll *a* fluorescence under increasing temperature (Schreiber and Berry 1977). Disks of tree-fern pinnules were placed into a temperature-controlled aluminium body, with the fibre-optics of the fluorometer (PAM 2000) pointing at the sample. Ground fluorescence (F_o) was induced with a red diode at low PPFD of ~1 µmol m^{-2} s^{-1}. Temperature of the aluminium body was increased gradually (1°C min^{-1}) from 20°C to 60°C. F_o was continuously recorded and critical temperature (T_c) was estimated graphically at the beginning of the heat-induced fluorescence rise (Froux *et al.* 2004).

Total tissue osmolality

Total tissue osmolality was measured using freeze-point depression from hot water extracts of dried frond tissue (Callister *et al.* 2006). Approximately 60 mg of dried ground frond tissue (as prepared for N analysis) was placed in a 2-mL polypropylene vial to which 1.6 mL of deionised water was

added. The samples were placed in a water bath at 90°C for 60 min. The samples were left to cool to room temperature, centrifuged at 10 000*g* for 2 min and 1 mL of the supernatant was transferred to a 1.7-mL polypropylene vial. Osmolality of the solution was measured using an OSMOMAT 030 cryoscopic osmometer (Gonotec, Berlin, Germany).

Pigments and tocopherol determination

Frond discs (3.75 mm diameter) were collected at midday at the end of each of the three temperature treatments and immediately frozen in liquid nitrogen. Samples were freeze-dried, sealed with silicagel in airtight plastic bags, and kept at −20°C until analysis.

Four discs per plant were ground in a Matrix Mill (Retsch MM301, Germany) at the temperature of liquid nitrogen. To avoid the presence of traces of acid in the acetone used for the extraction, 0.5 g L^{-1} of calcium carbonate were added to samples before grinding (García-Plazaola and Becerril 1999). The resulting powder was extracted with 0.5 mL of ice-cold acetone, homogenised and centrifuged at 4°C for 1 min at 15 000*g*. The pellets were re-extracted as described above to a combined sample volume of 1 mL. Extracts were stored in sealed vials at −20°C until analysis. Prior to injection, samples were centrifuged at 4°C for 20 min at 15 000 *g* and the clean supernatant was transferred into HPLC vials.

HPLC separation of chloroplast pigments and tocopherols was according to the methods given in Tausz *et al*. (2003). Chromatographic conditions were:

- Pigments: 25 × 4.6 mm Spherisorb ODS 25 µm column. gradient: solvent A: acetonitrile: methanol: water = 100 : 5 : 10 (v/v/v), solvent B: ethylacetate: acetone = 1 : 2 (v/v), 10% B to 70% B in 17 min, hold at 70% B for 5 min, return to 10% B in 5 min. Flow rate 1 mL min^{-1}. The injection volume was 20 µL, photometric detection at 440 nm.
- α-Tocopherol: 25 × 4.6 mm Spherisorb ODS 25 µm column. Solvent 100% methanol isocratic. Flow rate 1 mL min^{-1}. Injection volume was 20 µL. Fluorescence detection excitation 295 nm, emission 325 nm.
- Acetone, acetonitrile, methanol and ethyl acetate were of HPLC grade and water was deionised. A standard of α-tocopherol was purchased from Sigma (Sigma-Aldrich, Castle Hill, NSW, Australia); standards for carotenoids and chlorophyll *a* and *b* were prepared as follows: several generic extracts were prepared in 100% acetone and measured at three wavelengths in the spectrophotometer (Varian UV/V 300, USA). Using the equations of Lichtenthaler (1987), chlorophyll *a*, *b* and total carotenoid concentrations were calculated at a spectrophotometer resolution range of 1–4 nm. The same extracts were then re-run in the HPLC and conversion factors for chlorophyll *a*, *b* and total carotenoids were calculated, disregarding the minor differences in carotenoid absorption coefficients at the wavelength in question.

Statistical analysis

Repeated-measures models of SPSS 15 (SPSS Inc., Chicago, USA) were used for statistical analysis, with irradiance as the between-subject factor and temperature as the within-subject factor (both fixed). Effects of irradiance (high irradiance, shade), and 3 levels of temperature (15°C, 35°C, back to 15°C), and irradiance by temperature interactions on each dependent variable were analysed. Data for statistical analyses were the values per individual plant at the end of each temperature treatment. Photosynthetic parameters, measured before the start of the experiment (i.e. A_{max}, g_s, V_{cmax} and J_{max}) were not used in the model.

Results

Frond temperature (T_{frond})

T_{frond} did not differ among plants at the beginning of the experiment (data not shown). After the temperature increased to 35°C, T_{frond} was similar in shaded and high irradiance plants at predawn (below 25°C) but differed on average by 1.5°C at midday (around 35°C *v.* 33.5°C; Table 1). By the end of the treatment at 35°C, T_{frond} of shaded plants was on average 0.7°C cooler at predawn, and 3.3°C cooler at midday (Table 1).

Maximum quantum yield of PS II (F_v/F_m) and photosynthetic capacity parameters

Predawn F_v/F_m remained close to the optimum value of 0.83 in shaded plants across all temperature treatments. In contrast, F_v/F_m declined after the first day of exposure to high irradiance (Fig. 1*a*). The 35°C treatment resulted in further decreases of F_v/F_m (Fig. 1*a*). After return to 15°C, a partial recovery of F_v/F_m was detected.

Photosynthetic parameters were comparable among all plants at the start of the experiment (Table 2, 'before'). Ten days of high irradiance (at 15°C) resulted in decreases in both light-saturated

Table 1. Temperature of *Dicksonia antarctica* fronds (T_{frond}) exposed to high irradiance and under shade

Values are means ± s.e. (*n* = 7 plants, 10 measurements per plant) at predawn and midday on the first (day 1) and last (day 12) days of two temperature treatments (35°C and back to 15°C). *P*-values indicate significance of difference between high irradiance and shaded plants within temperature treatments (Student's *t*-test)

	35°C			*P*-value	Back to 15°C			*P*-value
	High irradiance	Shaded	Difference		High irradiance	Shaded	Difference	
				Predawn				
Day 1	24.3 ± 0.1	24.2 ± 0.1	0.1	0.30	15.3 ± 0.0	16.0 ± 0.0	–0.7	<0.001
Day 12	23.0 ± 0.2	22.3 ± 0.2	0.7	0.02				
				Midday				
Day 1	34.9 ± 0.6	33.4 ± 0.3	1.5	0.02	19.7 ± 0.4	17.2 ± 0.4	2.5	<0.001
Day 12	34.6 ± 0.2	31.3 ± 0.5	3.3	0.002				

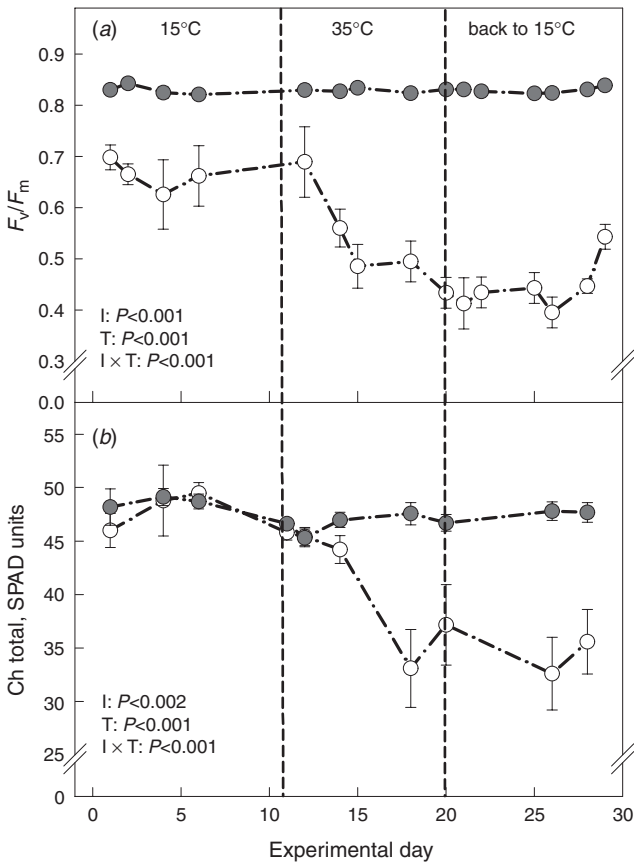

Fig. 1. Time course of (*a*) maximum quantum efficiency of PSII (F_v/F_m) recorded after 8 h of darkness (predawn); and (*b*) chlorophyll content in SPAD units of high irradiance (open symbols) and shaded (closed symbols) *Dicksonia antarctica* during three successive temperature treatments (delineated by dotted lines). Values are means of $n = 7$ (error bars indicate s.e.). *P*-values indicate significance of effects of irradiance (I), temperature (T), and irradiance by temperature interaction (I × T).

rate of net photosynthesis at a reference temperature of 25°C, A_{max}, and corresponding stomatal conductance g_s (Table 2; Fig. 2). Increasing the temperature to 35°C induced stomatal opening in all plants (Table 2). However, while A_{max} of all shaded plants also increased (Table 2; Fig. 2), A_{max} was less responsive in high irradiance plants (Fig. 2). Nonetheless, all changes in g_s and A_{max} were fully reversible upon return to 15°C (Table 2).

When measured at a reference temperature of 25°C, maximal carboxylation rate, $V_{cmax}{}^{25}$, and maximal light-driven electron flux $J_{max}{}^{25}$ decreased in response to increased irradiance. Both were insensitive to temperature treatments regardless of irradiance regime (Table 2). Mesophyll conductance to CO_2, g_i, was highly variable, probably owing to the low accuracy of the fitting procedure used. As a result, no significant effects of irradiance and temperature could be detected (Table 2).

Nitrogen content per frond area (g m^{-2}) was not affected by either irradiance or temperature (Table 2). Photosynthetic nitrogen use efficiency (PNUE), calculated as V_{cmax}/N, was lower under high irradiance than in shade and remained such until the end of the experiment. Changes in temperature did not affect PNUE (Table 2).

Total chlorophyll content (in SPAD units) decreased at 35°C under high irradiance and remained low thereafter (Fig. 1*b*). A similar albeit not significant effect was detected for chlorophyll *a* + *b* in HPLC extracts (Table 2). It should be noted that visually damaged fronds were avoided for HPLC analyses, while SPAD measurements were made across entire fronds. Chlorophyll *a*/*b* ratios were similar between irradiance regimes during the 15°C temperature treatment. With temperature increase to 35°C, chl *a*/*b* ratios decreased in high irradiance plants in contrast to a significant increase in shaded plants (Table 2). With temperature return to 15°C, chl *a*/*b* ratios of high irradiance plants recovered to the initial values.

Critical temperature (T_c)

During the 15°C treatment, critical temperature for photochemistry (T_c) was similar under shade and high irradiance (means of 47.5°C and 47.2°C, respectively; Fig. 3). Increase in temperature resulted in significant rises of T_c that were greatest under high irradiance. T_c started to decrease after return to 15°C, although pretreatment values were not reached by the end of the experiment (Fig. 3).

Total tissue osmolality

Total tissue osmolality was not affected by irradiance but decreased significantly during the 35°C treatment (Table 3). It increased to close to the original values after return to 15°C.

Carotenoids and α-tocopherol

Neoxanthin and lutein contents (per mol total chlorophyll) were significantly greater under high irradiance, whereas α- and β-carotene contents remained comparable to shaded plants (Table 3). Temperature increase to 35°C led to significant increase in β-carotene and lutein in high irradiance plants. These pigments tended to remain high on return to 15°C, although α-carotene significantly decreased under high irradiance (Table 3).

The xanthophyll cycle pigment pool (i.e. violaxanthin, antheraxanthin, and zeaxanthin, V+A+Z) was significantly greater under high irradiance for the whole experiment. Changes in temperature did not affect V+A+Z under either irradiance regime (Table 3). The de-epoxidation state of xanthophylls (expressed as (0.5A+Z)/(V+A+Z)) was greater under high irradiance than shade (on average 44% *v.* 3%; Table 3). It decreased by *c.* 32% under high irradiance upon return to 15°C, but was insensitive to temperature under shade. Small amounts of lutein-epoxide were detected (Table 3), but they were not affected by light, temperature and their interactions.

Content of α-tocopherol was significantly greater under high irradiance than shade (Fig. 4). Whereas α-tocopherol was invariant to temperature changes in shaded plants, α-tocopherol increased in high irradiance plants with temperature increase to 35°C, and remained high after the temperature returned to 15°C; due to the lack of a high light-low temperature control, we cannot clearly separate the high irradiance and high temperature effects on these changes.

Table 2. Photosynthesis and frond traits of *Dicksonia antarctica* exposed to high irradiance and under shade before and during three successive temperature treatments

Values are means ± s.e. ($n = 7$) of A_{max}^{25} – light-saturated net CO_2 assimilation rate at 25°C; g_s, stomatal conductance under saturating irradiance at 25°C; N_A, nitrogen content on a frond area basis; PNUE, photosynthetic nitrogen use efficiency (V_{cmax}/N_A) at 25°C; Chl total, total chlorophyll content in SPAD units and on a frond area basis; Chl *a*/*b*, chlorophyll *a*/*b* ratio; V_{cmax}^{25}, maximal carboxylation rate of Rubisco at 25°C; J_{max}^{25}, maximal light driven electron flux at 25°C; g_i, mesophyll conductance to CO_2 measured at 25°C; Abbreviations: I, Irradiance; T, temperature; I × T, irradiance by temperature interaction; n.s., not-significant; n.d., no data

Parameter	Irradiance regime	Temperature treatments				Significance of effects (*P*)		
		Before	15°C	35°C	Back to 15°C	I	T	I × T
A_{max}^{25} (μmol CO_2 m^{-2} s^{-1})	High irradiance	6.0 ± 0.5	3.7 ± 0.5	5.1 ± 0.6	3.9 ± 0.4	<0.001	0.02	n.s. (0.3)
	Shade	6.4 ± 0.4	6.4 ± 0.8	7.6 ± 0.3	5.6 ± 0.5			
g_s at A_{max}^{25} (mmol H_2O m^{-2} s^{-1})	High irradiance	82 ± 11	57 ± 6	108 ± 7	50 ± 6	0.02	<0.001	n.s. (0.3)
	Shade	80 ± 7	80 ± 14	152 ± 12	70 ± 5			
V_{cmax}^{25} (μmol m^{-2} s^{-1})	High irradiance	37.0 ± 2	29.3 ± 2.7	23.2 ± 2.8	29.1 ± 3.3	0.001	n.s. (0.7)	
	Shade	36.0 ± 1	37.1 ± 2.9	40.6 ± 3.8	34.5 ± 4.2			
J_{max}^{25} (μmol m^{-2} s^{-1})	High irradiance	115 ± 6	67.8 ± 8.7	50.2 ± 7.3	84.7 ± 10.8	0.01	n.s. (0.1)	
	Shade	105 ± 6	84.6 ± 10.2	85.6 ± 11.7	97.7 ± 9.8			
g_i (mmol CO_2 m^{-2} s^{-1})	High irradiance	155 ± 64	141 ± 53	143 ± 115	115 ± 25	n.s. (0.2)	n.s. (0.8)	
	Shade	115 ± 35	222 ± 63	294 ± 195	259 ± 112			
N_A (g m^{-2})	High irradiance	n.d.	16.9 ± 1.0	15.9 ± 0.6	17.4 ± 0.7	n.s. (0.9)	n.s. (0.4)	
	Shade	n.d.	16.5 ± 0.7	16.0 ± 0.8	15.5 ± 2.1			
PNUE (V_{cmax}/N_A) (μmol mol^{-1} N^{-1} m^{-2})	High irradiance	n.d.	204 ± 21	201 ± 24	175 ± 13	0.01	n.s. (0.4)	
	Shade	n.d.	278 ± 18	311 ± 30	300 ± 41			
Chl total (SPAD units)	High irradiance	n.d.	49.1 ± 1.7	39.3 ± 2.3	34.1 ± 2.1	0.002	<0.001	0.001
	Shade	n.d.	48.9 ± 0.4	47.1 ± 0.5	47.3 ± 1.0			
Chl total (*a* + *b*) (μmol m^{-2})	High irradiance	n.d.	782 ± 142	721 ± 111	675 ± 89.6	n.s. (0.2)	n.s. (0.8)	
	Shade	n.d.	810 ± 62.7	914 ± 127	843 ± 81.1			
Chl *a*/*b*	High irradiance	n.d.	2.36 ± 0.06	2.29 ± 0.05	2.41 ± 0.09	0.002	0.005	<0.001
	Shade	n.d.	2.46 ± 0.04	2.71 ± 0.05	2.66 ± 0.03			

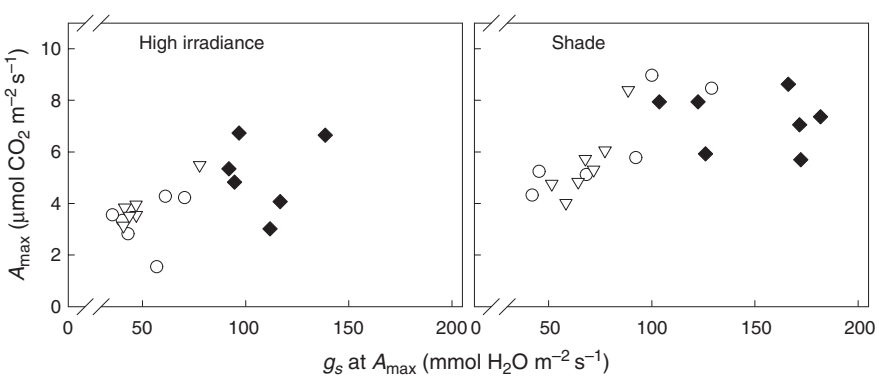

Fig. 2. Stomatal conductance (g_s) at A_{max} *v.* light-saturated rate of net photosynthesis (A_{max}) for high irradiance and shaded *Dicksonia antarctica* measured at a standardised temperature of 25°C during three temperature treatments: 15°C (open circle), 35°C (closed diamond) and back to 15°C (open triangle). Values are means of $n = 6$ (high irradiance) and $n = 7$ (shaded).

Correlations between T_c *and biochemical parameters*

No significant correlation was detected between T_c and osmolality, tocopherol, and α- and β-carotenes (correlations not shown). Correlations between T_c and zeaxanthin, presented as Z/(Z+V) (after Havaux and Gruszecki 1993) were also not significant, either for each irradiance treatment separately or for the combined data (Fig. 5).

Discussion

Effect of high irradiance, high temperature and their interaction on photosynthetic capacity parameters of D. antarctica

Photosynthetic capacity of *Dicksonia antarctica* in this study was within ranges reported in the literature. Maximum light saturated rates of net photosynthesis (A_{max}^{25}) were comparable with values

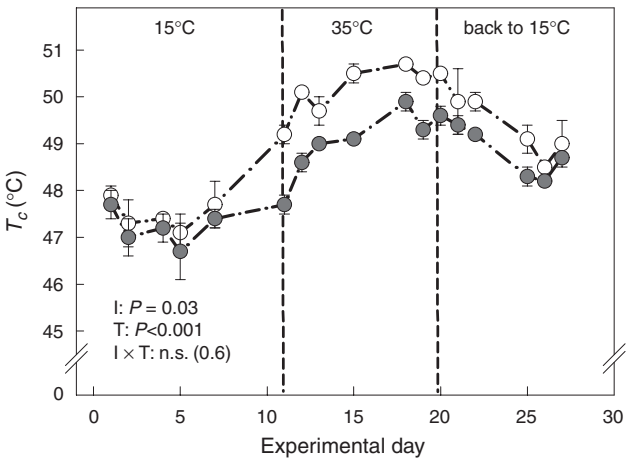

Fig. 3. Time course of critical temperature (T_c) of high irradiance (open symbols) and shaded (closed symbols) *Dicksonia antarctica* across the experiment. Values are means of $n = 7$ (±s.e.). *P*-values indicate significance of effects of irradiance (I), temperature (T), and irradiance by temperature interaction (I × T).

reported for the same species: 6 – 10.8 μmol m^{-2} s^{-1} (Nobel *et al.* 1984) and 8.3 μmol m^{-2} s^{-1} (Hunt *et al.* 2002). Maximum carboxylation rates, V_{cmax}^{25} (the maximal *in vivo* Rubisco activity), and the maximum rate of electron transport, $J_{max,}$ at a reference temperature of 25°C were comparable to some shade tolerant tree species, such as silver fir (*Abies alba* Mill; Robakowski *et al.* 2002), and mesophyll conductance, g_i, corresponded to typical values of evergreen trees among the species reviewed by Ethier and Livingston (2004).

Photosynthetic capacity of *D. antarctica* was adversely affected by high irradiance. J_{max}^{25} decreased with respect to shaded plants, and this decrease was paralleled by a decrease of maximum quantum yield of PSII, F_v/F_m, indicating a moderate but chronic photoinhibition (Table 2; Tallon and Quiles 2007). High irradiance also led to decreases in A_{max}^{25}, due to both reduced stomatal conductance g_s and decreased photosynthetic capacity, i.e. Rubisco activity and J_{max}^{25}. Several processes may result in deactivation of Rubisco – for example, remobilisation and export of nitrogen from the leaves, interruption in the electron transport chain, or the presence of reactive oxygen species (ROS). In our case, deactivation of Rubisco in high irradiance plants was not related to a remobilisation of frond nitrogen – the N content per frond area was not affected by irradiance. This can be seen also through the decline of photosynthetic nitrogen use efficiency (PNUE). However, high irradiance alone did not affect total chlorophyll content and chlorophyll *a*/*b* ratios, indicating that photoprotective mechanisms were efficient enough to avoid chlorophyll degradation.

High temperature had no negative effects on photosynthetic capacity of *D. antarctica* under shade. Increasing temperature to 35°C even stimulated A_{max}^{25}. Increases in A_{max}^{25} were in this case solely due to increases in g_s, as we found no effects of temperature on photosynthetic capacity (V_{cmax}^{25}, J_{max}^{25}). The increase in chlorophyll *a*/*b* ratio in shaded plants with increasing temperature perhaps indicates temperature-stimulated resynthesis of photosynthetic reaction centres relative to light-harvesting antenna complexes, which commonly coincides with other changes in pigment composition, e.g. lutein, β-carotene (Haldimann 1999).

The interactive effect of high irradiance and high temperature led to severe photoinhibition in agreement with earlier findings (Berry and Björkman 1980). Temperature increase stimulated g_s yet without commensurate increases in A_{max}^{25}, indicating that metabolic limitations (e.g. Rubisco activity) governed A_{max}^{25} (consistent with the findings of e.g. Law and Crafts-Brandner 1999; on Rubisco activation). Many studies have shown a

Table 3. Pigment content and osmolality of *Dicksonia antarctica* fronds exposed to high irradiance and under shade during three successive temperature treatments

Values are means ± s.e. ($n = 7$) of: osmolality; carotenoids (on a chlorophyll basis): lutein, neoxanthin and xanthophyll pool (i.e. Violanxanthin, Antheraxanthin, Zeaxanthin, V+A+Z), α- and β-carotene, lutein-epoxide and the de-epoxidation state of violaxanthin = (0.5A+Z)/(V+A+Z). Abbreviations: I, Irradiance; T, temperature; I × T, irradiance by temperature interaction; n.s., not significant

	Irradiance regime	Temperature treatments			Significance of effects (*P*)		
		15°C	35°C	Back to 15°C	I	T	I × T
Osmolality (mosmol g d.w.$^{-1}$)	High irradiance	1.56 ± 0.06	1.33 ± 0.04	1.45 ± 0.05	n.s. (0.4)	<0.001	
	Shade	1.55 ± 0.02	1.40 ± 0.03	1.51 ± 0.01			
V+A+Z (mmol mol^{-1} chl^{-1})	High irradiance	91.9 ± 9.2	118.1 ± 15.0	113.7 ± 15.9	<0.001	n.s. (0.2)	
	Shade	47.8 ± 1.7	45.8 ± 1.2	45.5 ± 1.2			
De-epoxidation (%)	High irradiance	56.6 ± 3.4	53.1 ± 7.7	21.4 ± 4.7	<0.001	<0.001	0.001
	Shade	4.4 ± 1.1	2.3 ± 0.8	1.6 ± 0.6			
Lutein (mmol mol^{-1} chl^{-1})	High irradiance	263 ± 9.0	339 ± 25.4	388 ± 28.5	<0.001	0.002	0.008
	Shade	185 ± 3.9	186 ± 3.3	198 ± 5.9			
Neoxanthin (mmol mol^{-1} chl^{-1})	High irradiance	61.3 ± 2.6	65.9 ± 3.8	69.3 ± 3.6	<0.001	n.s. (0.06)	
	Shade	45.2 ± 1.9	46.2 ± 1.1	48.4 ± 2.0			
α-carotene (mmol mol^{-1} chl^{-1})	High irradiance	11.1 ± 2.3	24.2 ± 3.4	5.8 ± 1.5	0.03	<0.001	0.003
	Shade	16.1 ± 2.6	24.1 ± 2.4	20.9 ± 1.8			
β-carotene (mmol mol^{-1} chl^{-1})	High irradiance	62.2 ± 7.8	94.6 ± 8.7	89.3 ± 7.0	0.001	0.005	n.s. (0.4)
	Shade	44.7 ± 5.7	59.4 ± 5.3	59.3 ± 3.3			
Lutein-epoxide (mmol mol^{-1} chl^{-1})	High irradiance	5.2 ± 1.7	6.3 ± 1.4	6.2 ± 2.2	n.s. (0.1)	n.s. (0.2)	
	Shade	3.4 ± 0.4	3.9 ± 0.4	4.3 ± 0.3			

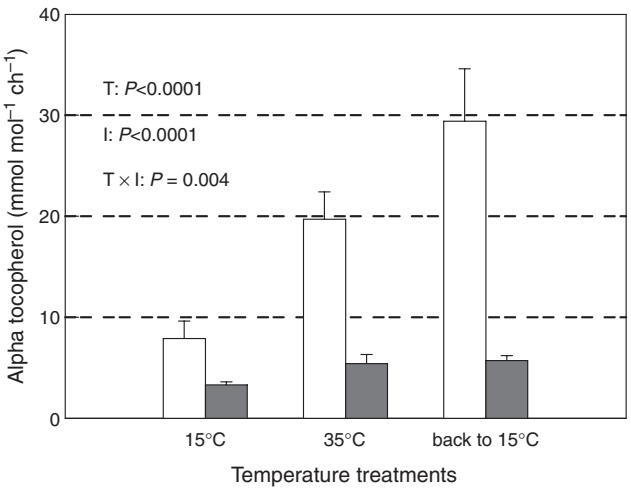

Fig. 4. α-Tocopherol content relative to total chlorophylls of high irradiance (open bars) and shaded (closed bars) *Dicksonia antarctica* under three temperature treatments. Values are means of $n = 7$ (±s.e.). *P*-values indicate significance of effects of irradiance (I), temperature (T), and irradiance by temperature interaction (I × T).

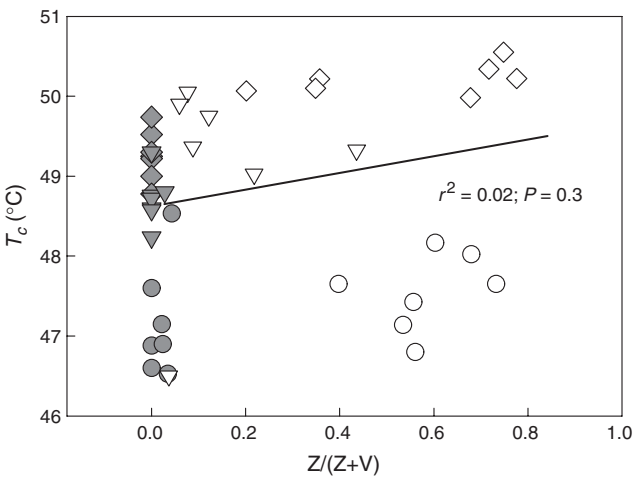

Fig. 5. Critical temperature (T_c) *v.* xanthophyll zeaxanthin (expressed as Z/(Z+V); after Havaux and Gruszecki 1993) of high irradiance (open symbols) and shaded (closed symbols) *Dicksonia antarctica* during three temperature treatments: 15°C (circle), 35°C (diamond) and back to 15°C (triangle). Each point represents an individual measurement. Relationships were also non-significant when data were separated by irradiance treatment. Z, zeaxanthin; V, violaxanthin.

negative effect of moderate heat and photoinhibition on the activation of Rubisco mediated by an activase (for details, see Salvucci and Crafts-Brandner 2004). In our study, we did not measure the activity of Rubisco-activase, but found no increase in photosynthetic capacity to increased temperatures. Prolonged photoinhibition and heat interaction resulted in decreases in chlorophylls as in numerous other studies (e.g. Lambers *et al.* 2008). Decreases in chlorophyll *a*/*b* ratio indicated that under prolonged light stress, chlorophylls were destabilised with Chl *a* being more sensitive than Chl *b* (Yamamoto *et al.* 2008).

A return of temperature to 15°C induced stomatal closure thus reversing A_{max}[25] to the initial values at 15°C, as found by Ghouil *et al.* (2003). The only partial recovery of F_v/F_m in high irradiance plants demonstrated detrimental interactive effects of high irradiance and temperature on F_v/F_m, suggesting that degradation processes (i.e. bleaching of chlorophylls or photodegradation of thylakoid complexes) did not allow rapid recovery of F_v/F_m (Ottander *et al.* 1995). The fact that V_{cmax} and J_{max} remained non-responsive possibly underlined the high sensitivity of Rubisco to high irradiance in *D. antarctica*.

In summary, photosynthetic capacity and photosynthetic nitrogen use efficiency were rapidly affected by exposure to high irradiance under 15°C, while chlorophylls and predawn F_v/F_m declined further under the combination of high irradiance and high temperature.

Membrane stability of D. antarctica *measured via critical temperature*

Critical temperature, T_c, recorded in *D. antarctica* was *c.* 47°C, comparable with a range of overstorey species, such as *Quercus petraea* Matt. Liebl. (46.7°C; Dreyer *et al.* 2001). Comparable data for other tree-ferns species are currently lacking.

An increase in air temperature induced an increase in T_c, as found by many authors (e.g. Dreyer *et al.* 2001). This increase in T_c was larger under high irradiance than shade, coinciding with a 3.3°C higher midday frond temperature under high irradiance. Previously published data suggest that an increase in T_c by 1°C (as found in our study) requires an increase in ambient temperatures of ~10°C (Froux *et al.* 2004). We therefore believe that the larger rise in T_c in high irradiance plants was not solely caused by the difference in frond temperature, but directly related to high irradiance effects, which resulted in enhanced thermostability of thylakoid membranes.

Contrary to findings by Hüve *et al.* (2006), increased thermostability of the thylakoid membranes was not associated with the accumulation of osmotically active substances. In our study, osmotically active solutes even decreased when temperature increased. It may be argued that the hot water extract method results in artefacts, because some cell wall or other material can be brought into solution as a result of the grinding and extraction procedures (Callister *et al.* 2006). Yet this method is widely used (e.g. Merchant *et al.* 2006) and moreover, a tight correlation was found among methods even though the absolute results were different (Callister *et al.* 2006). Our observation therefore seems reliable.

With subsequent temperature decrease to 15°C, we found a tendency for T_c to return to the initial values, even though this return was not complete. The observed lag confirms the hysteresis found by Froux *et al.* (2004), when the increase in T_c with increasing temperature is faster than relaxation from this effect after temperature decrease.

Xanthophyll cycle carotenoids, pigments and α-tocopherol

Exposure of *D. antarctica* to high irradiance alone resulted in almost 2-fold increase in xanthophyll cycle carotenoids (V+A+Z). A high de-epoxidation state was also recorded

(50% *v.* 2–4% in the shade). Values for V+A+Z were within the range of values presented for other species (Thayer and Björkman 1990). The maximum de-epoxidation state of the xanthophyll cycle was lower than maximum values measured in epiphytic ferns (Tausz *et al*. 2001). An increase was also recorded in pools of other carotenoids such as neoxanthin and lutein in response to high irradiance. Values were within the range reported for other ferns, e.g. a range of epiphytic species (Tausz *et al*. 2001) or the tree-fern *Cyathea microdonta* (Desv.) Domin (Matsubara *et al*. 2009). These carotenoids (e.g. neoxanthin) may preserve PSII from photo-inactivation and protect membrane lipids from photo-oxidation by ROS (North *et al*. 2007). The observed increase in lutein (located primarily in both the proximal and distal light harvesting centres of PSI and PSII) is probably associated with the acclimation of antennae to increasing irradiance (Senger *et al*. 1993). As antenna size is usually reduced in response to increasing irradiance, it is also likely that an increasing fraction of these carotenoids is not bound to antenna proteins. High irradiance also induced increases in α-tocopherols, known for their protective function in thylakoids, consistent with findings elsewhere (e.g. García-Plazaola and Becerril 1999; Munné-Bosch 2005).

Increased temperature stimulated an increase in T_c in shaded plants but without simultaneous accumulation of zeaxanthin, which is contrary to observations by Havaux and Gruszecki (1993) and Havaux and Tardy (1996). Irrespective of an increased T_c, high temperature had no effect on concentration of α-tocopherol in shaded plants. According to our results, it is therefore unlikely that increased membrane stability as measured by T_c is directly and generally dependent on zeaxanthin or α-tocopherol. There was some coincidence of increased T_c with increases in α- and β-carotenes in shaded plants, which may have reflected changes in carotenoid synthesis rates. Carotenes may also contribute to an overall increase in membrane stability, as proposed by Tausz *et al*. (2001).

Interactions between irradiance and temperature had no additional effect on the xanthophyll cycle pool and de-epoxidation state of high irradiance plants. In contrast to V+A+Z, the combination of high irradiance with high temperature led to further increases in the amount of lutein and β-carotene, which remained greater until the end of experiment. It is not clear from our experiment whether sustained concentrations of carotenoids indicated their role as a last resort in membrane photoprotection under extreme stress, or simply reflected their superior stability under such conditions. In contrast to shaded plants, temperature increase stimulated an almost 2-fold increase in α-tocopherol in high irradiance plants. Although these results for high irradiance plants appear to support findings of Llusià *et al*. (2005) – who suggested that increased tolerance to high temperatures might be at least partly due to an increase in α-tocopherol – our results for shaded plants indicate that changes in T_c can occur independently of changes in α-tocopherol.

Temperature return to 15°C did not affect total V+A+Z pool of high irradiance plants, but the de-epoxidation state decreased by 30% to remain significantly higher than in shaded plants. De-epoxidation of the xanthophyll cycle is driven by an acidic thylakoid lumen, which can be the consequence of an imbalance between electron transport and electron consumption (Demmig-Adams and Adams 2006). It seems that heat related changes in the photosynthetic apparatus lead to a relaxation of the pH gradient upon temperature decrease, despite the continuation of high irradiance. This may be related to a sustained decrease in light use efficiency as suggested by persistently low F_v/F_m values, and other, as yet unexplained, changes in the photosynthetic membrane. Such further changes were also expressed in the observed change in T_c, possibly in combination with an increased electron consumption rate upon relaxation of the high temperature. Temperature decrease did not affect concentration of carotenoids except for α-carotene. Significant decreases in α-carotene in high irradiance plants can be explained in terms of its ease of oxidation to lutein under conditions of oxidative stress (Senger *et al*. 1993), or its conversion to β-carotene to increase scavenging of free radicals in core complexes under conditions of stress (Kirchgeßner *et al*. 2003).

In summary, increased thylakoid stability in *D. antarctica* observed during our experiment could not be explained by any of the measured changes in pigments or α-tocopherol, although they may all play partial roles. Alternative or additional explanations may involve presence of certain heat shock proteins or changes in the composition of membrane lipids, as suggested by Sinsawat *et al*. (2004). Discrepancies with earlier literature may be related to the fact that pigment changes are fast responses to temperature increases (in the order of 1 day; e.g. as in Havaux and Tardy 1996), while we investigated longer term acclimation (12 days) to high temperature. Longer term acclimation of plants to rising temperatures is also related to the appearance of polar lipids with saturated fatty acids causing a decrease in membrane fluidity (Zsófi *et al*. 2009). In parallel with an increased threshold temperature for thermal inactivation of PSII (Downton *et al*. 1984), this can increase thylakoid stability. The difference between the rate of acclimation and de-acclimation supports this hypothesis: the fast rise may be initially due to rapid changes in pigments that are later completed by slower changes in lipid composition. The reversal of these changes may be slower, which would be the cause for a slow return to initial levels of stability. However, our data do not allow confirmation of this speculation.

Conclusion

High irradiance caused chronic photoinhibition (measured as sustained decrease in maximum PSII quantum efficiency), and decreases in all photosynthetic capacity parameters of *D. antarctica.* Whilst we observed some acclimation in terms of increases in protective carotenoids, which may have sufficed to avoid chlorophyll degradation, similar or even decreasing chlorophyll *a*/*b* ratios indicated limited short-term acclimation potential of *D. antarctica* fronds to high irradiance. Temperature alone appeared to have no negative effect on photosynthesis of *D. antarctica*, possibly suggesting that this species can thrive under moderately high temperature as long as it is shaded. However, there was ample evidence of severe damage to the photosynthetic apparatus when high irradiance was combined with moderately high temperatures. Therefore we can speculate that future scenarios predicting higher temperatures and more frequent disturbances may decrease the competitiveness of *D. antarctica*.

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