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Photosynthetic limitations and volatile and non-volatile isoprenoids in the poikilochlorophyllous resurrection plant *Xerophyta humilis* during dehydration and rehydration

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ABSTRACT

We investigated the photosynthetic limitations occurring during dehydration and rehydration of *Xerophyta humilis*, a poikilochlorophyllous resurrection plant, and whether volatile and non-volatile isoprenoids might be involved in desiccation tolerance. Photosynthesis declined rapidly after dehydration below 85% relative water content (RWC). Raising intercellular CO_2 concentrations during desiccation suggest that the main photosynthetic limitation was photochemical, affecting energy-dependent RuBP regeneration. Imaging fluorescence confirmed that both the number of photosystem II (PSII) functional reaction centres and their efficiency were impaired under progressive dehydration, and revealed the occurrence of heterogeneous photosynthesis during desiccation, being the basal leaf area more resistant to the stress. Full recovery in photosynthetic parameters occurred on rehydration, confirming that photosynthetic limitations were fully reversible and that no permanent damage occurred. During desiccation, zeaxanthin and lutein increased only when photosynthesis had ceased, implying that these isoprenoids do not directly scavenge reactive oxygen species, but rather protect photosynthetic membranes from damage and consequent denaturation. *X. humilis* was found to emit isoprene, a volatile isoprenoid that acts as a membrane strengthener in plants. Isoprene emission was stimulated by drought and peaked at 80% RWC. We surmise that isoprene and non-volatile isoprenoids cooperate in reducing membrane damage in *X. humilis*, isoprene being effective when desiccation is moderate while non-volatile isoprenoids operate when water deficit is more extreme.

Key-words: carotenoids; desiccation tolerance; imaging fluorescence; isoprene; photosynthesis; volatile organic compounds.

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INTRODUCTION

Desiccation tolerance is defined as the ability of an organism to dry to equilibrium with the air dry state and to resume full metabolic function on rehydration. Although near complete measurable water loss is a common occurrence in the development of seeds and pollen (reviewed in Berjak, Farrant & Pammenter 2007) and in a large number of lichen and bryophyte species, there are only a few higher order plants that are able to survive extreme water deficit in their vegetative tissues (reviewed in Farrant 2007; Oliver 2007; Moore *et al*. 2009). Desiccation tolerance has only been observed in about 350 angiosperm species, termed 'resurrection plants', the majority of which are found in tropical and subtropical zones in southern Africa.

In vegetative tissues, severe water stress results in a disruption of electron transport and therefore disequilibrium between reactive oxygen species (ROS) production and scavenging ensues (Halliwell 1987; Halliwell & Gutteridge 1999; Apel & Hirt 2004). Not only there is an excess of ROS produced from respiratory metabolism, but also from a disruption of photosynthesis and consequent inefficient use of light-generated photosynthetic electron transport. Excess energy from excited chlorophyll molecules is transferred to oxygen causing a rapid production of free radical species. While desiccation-sensitive plants are unable to adequately deal with the surge in ROS production, which ultimately leads to cell death (Smirnoff 1993; Kranner & Birtić 2005), resurrection plants have many mechanisms in place to firstly reduce ROS formation and, secondly, quench their activity (reviewed in Farrant 2007; Farrant, Cooper & Nell 2012).

Photosynthetic ROS production is minimized in resurrection plants at high relative water contents (RWCs), this being accomplished by one of two mechanisms termed poikilochlorophylly and homoiochlorophylly (Gaff 1989; Sherwin & Farrant 1998; Tuba, Proctor & Csintalan 1998; Farrant 2000, 2007). Poikilochlorophylly involves the breakdown of chlorophyll and partial dismantling of thylakoid membranes during dehydration, resulting in a cessation of

photosynthesis at water contents between 80 and 65% in species tested to date. Homoiochlorophyllous resurrection plants retain chlorophyll and thylakoid membranes in the desiccated state but light–chlorophyll interactions that initiate photosynthesis and thus ROS production are minimized by a number of adaptive mechanisms. These include leaf folding to reduce the surface area exposed to light and heat, the presence of reflective hairs and/or waxes, and the accumulation of anthocyanins or other pigments that mask chlorophyll and act as antioxidants (reviewed in Farrant 2007; Farrant *et al*. 2012).

Clearly, there is a very controlled regulation of physical and metabolic processes in resurrection plants that enables minimization of the stresses associated with desiccation and that allows full recovery when water becomes available. These adaptations separate resurrection plants from desiccation-sensitive plants.

Non-volatile isoprenoids, such as tocopherols, zeaxanthin and β-carotene, form an integral part of the non-enzymatic oxidative defence system in all plants (Demmig-Adams & Adams III 1996; Munné-Bosch & Alegre 2002; Telfer 2002). Recently, carotenoids, particularly zeaxanthin, have been reported to enhance drought tolerance (Davison, Hunter & Horton 2002; Du *et al*. 2010), not only by quenching the singlet oxygen-excited state of chlorophyll or through non-photochemical quenching (NPQ), but also exerting a specific role in preserving thylakoid membranes from peroxidation (Havaux & Niyogi 1999; Havaux, Dall'Osto & Bassi 2007), a function that cannot be served by other non-volatile isoprenoids, such as α-tocopherol and β-carotene (Havaux *et al*. 2007).

There are various other molecules that function during drought stress in some desiccation-sensitive plants, which have not yet been investigated in resurrection plants. Among these are volatile organic compounds (VOCs). Isoprene (C_5H_8, 2-methyl-1,3-butadiene) is a natural product of many organisms and is the most abundant VOC emitted by terrestrial plants (Guenther *et al*. 1995). During environmental stress conditions such as drought stress or elevated temperature, the amount of carbon lost due to isoprene emission (regularly limited to 1–2% of photosynthesis) can increase dramatically (Sharkey & Loreto 1993). Many plants from a broad range of taxonomic groups emit isoprene, such as mosses, ferns, gymnosperms and angiosperms (Tingey *et al*. 1987; Hanson *et al*. 1999; Sharkey *et al*. 2005). However, there are also many members from these groups that do not emit isoprene. As the energy cost of isoprene emission is significant, especially under stress conditions (Sharkey, Chen & Yeh 2001; Sharkey, Wiberley & Donohue 2008), it is probable that benefit outweighs the cost in the plants in which isoprene emission occurs. Researchers have therefore been intent on determining and investigating the benefits that isoprene confers on emitting plants.

To date, the best characterized roles of isoprene have been in thermotolerance (Sharkey & Yeh 2001; Sharkey *et al*. 2008; Velikova *et al*. 2011) and ozone stress (Loreto *et al*. 2001; Loreto & Velikova 2001). Isoprene has been shown to protect the photosynthetic apparatus against ozone damage, quench ozone products, such as hydrogen peroxide, and help reduce lipid peroxidation of membranes. While isoprene has been proposed to operate a protective action also under drought stress conditions (Sharkey & Loreto 1993; Fortunati *et al*. 2008), there have been no reports on its role in plants that tolerate extreme water deficit stress.

Volatile isoprenoids, such as isoprene, may form part of an additional protective system against oxidative stress, which is not conserved among all plants. While, as discussed above, many plants emit isoprene, this work has mainly been reported for desiccation-sensitive woody plants (Vickers *et al*. 2009), and it is not known whether resurrection plants emit volatile isoprenoids. As such, emission seems to convey an adaptive advantage to plants to survive in adverse environmental conditions, investigating VOCs in resurrection plants could uncover an additional mechanism in desiccation tolerance.

The aim of this study was firstly to investigate photosynthetic regulation and some of the known conserved antioxidant processes during dehydration of the poikilochlorophyllous resurrection plant *Xerophyta humilis*, and, thereafter, to investigate whether this species emits VOCs and to characterize their role in acquisition of desiccation tolerance.

MATERIALS AND METHODS

Plant material

X. humilis plants were collected in the Pilanesberg Nature Reserve, South Africa, and maintained in a glasshouse as previously described (Sherwin & Farrant 1996). Plants were then dehydrated and transported to Italy in the desiccated state. On arrival, they were rehydrated and maintained in a climatized glasshouse and allowed to acclimatize for 3 weeks at a temperature maintained above 25 °C and variable during the experiment between 25 and 31 °C, and a light intensity reaching 700 µmol m^{-2} s^{-1} during sunny days prior to conducting the experiments described below. Trays (15 × 20 cm with a soil depth of about 5 cm) containing up to 15 plants were used for the procedures described below.

RWC determination

Trays were well watered to ensure plants were fully hydrated at commencement of an experiment. Thereafter, whole plants were dehydrated by withholding water, and allowing plants to dry naturally. Soil was watered to field capacity to allow for rehydration.

The water content was obtained gravimetrically on a dry weight (DW) basis by oven drying at 70 °C for 48 h. RWC was measured using the standard formula: RWC = water content/water content at full turgor and was expressed as a percentage. Full turgor was achieved as previously described (Mowla *et al*. 2002). Three leaf sections randomly chosen from plants within each tray were cut for RWC determination.

Measurement of chlorophyll fluorescence

A Maxi-Imaging-PAM-fluorometer (Heinz Walz GmbH, Effeltrich, Germany) was used for chlorophyll *a* fluorescence measurements. The MAXI version of the IMAGING-PAM M-Series employs a very compact and powerful 300W LED array for homogeneous illumination of up to 10×13 cm areas with pulse-modulated excitation, actinic light and saturation pulses. The charge-coupled device (CCD) camera has a resolution of 640×480 pixels. Pixel value images of the fluorescence parameters were displayed using a false colour code ranging from black (0.000) to red, yellow, green, blue and pink (1.000).

Plants were dark adapted for at least 20–35 min prior to the determination of F_o and F_m (minimum and maximum fluorescence, respectively). Longer dark-adaptation time courses were used as plants underwent dehydration to compensate for a slightly delayed relaxation of the reaction centres of photosystem II (PSII). The maximum quantum yield of PSII photochemistry (F_v/F_m) was determined as $(F_m - F_o)/F_m$. Leaves were adapted to the specific light level and a saturating pulse of 0.8 s with 6000 μmol photons m^{-2} s^{-1} was applied in order to determine the maximum fluorescence (F'_m) and the steady-state fluorescence (F_s) during the actinic illumination. It was tested along the dehydration that the light intensity was sufficient to saturate PSII and that the length of the pulse did not induce photoinhibition. The quantum efficiency of PSII photochemistry, Φ_{PSII}, was calculated using the formula: $(F'_m - F_s)/F'_m$ (Genty, Briantais & Baker 1989). The coefficient of photochemical quenching, qP, is a measurement of the fraction of open centres calculated as $(F'_m - F_s)/(F'_m - F'_o)$ (Schreiber, Schliwa & Bilger 1986). The value of F'_o was estimated using the approximation: $F'_o = F_o/(F_v/F_m + F_o/F'_m)$ (Oxborough & Baker 1997). Calculation of quenching due to non-photochemical dissipation of absorbed light energy (NPQ) was determined using the equation $NPQ = (F_m - F'_m)/F'_m$ (Bilger & Björkman 1991). Chlorophyll fluorescence determinations were obtained from $n = 7$ leaves, selected on the images of whole plant parts that were reached by an incident light intensity of 610 μmol m^{-2} s^{-1}, as this was the light intensity closest to growth light intensity (see below).

Pigment analyses

Individual carotenoids were identified and quantified as reported in García-Plazaola & Becerril (1999). Fresh leaf material (120–150 mg) was extracted with 2×4 mL acetone (added with 0.5 g L^{-1} $CaCO_3$) and 15 μL aliquots were injected in a Perkin Elmer Flexar chromatograph equipped with a quaternary 200Q/410 pump and LC 200 diode array detector (DAD) (all from Perkin Elmer, Bradford, CT, USA).

Photosynthetic pigments were separated by a 250×4.6 mm Waters Spherisorb ODS1 (5 μm) column operating at 30 °C, eluted with a linear gradient solvent system, at a flow rate of 1.2 mL min^{-1} consisting of CH_3CN/MeOH/H_2O (8.4/0.8/0.7, A) and MeOH/ethyl acetate (6.8/3.2, B) during an 18 min run: 0–12 min from 100 to 0% A; 12–18 min at 0% A.

Violaxanthin cycle pigments, lutein and α-, β-carotene were identified using visible spectral characteristics and retention times. The compounds were calibrated as such: neoxanthin, violaxanthin and antheraxanthin with the calibration curve of lutein at lower concentration points; lutein with the calibration curve of lutein; zeaxanthin with the calibration curve of zeaxanthin; and α-carotene and β-carotene with the calibration curve of β-carotene (all from Extrasynthese, Lyon-Nord, Genay, France). Chlorophyll *a* and *b* were quantified by spectrophotometric analysis (Lichtenthaler & Buschmann 2001). The dehydration-rehydration experiment was repeated twice and at least three biological replicates were obtained for each RWC point plotted.

Photosynthesis and VOC measurements

Gas exchange and VOC emission measurements were performed simultaneously on leaves of *X. humilis* grown in soil. Plants were transferred to the laboratory for the duration of the measurement and kept under a light source with an intensity of 700 μmol photons m^{-2} s^{-1}, equivalent to the mean light intensity in the greenhouse during daylight. Leaves were marked with small adhesive labels and the same leaves were followed during dehydration and rehydration. The experiment was repeated three times and at least four biological repeats (different leaves from different plants) were obtained for each RWC plotted.

Rates of photosynthesis (A) and photosynthesis response to intercellular CO_2 concentration (A/C_i responses) were measured on a 2 cm^2 leaf piece flattened in the cuvette of a LI-6400 (Li-Cor Biosciences Inc., NE, USA) infrared gas analyser (IRGA) allowing simultaneous measurements of gas exchange and florescence on the same leaf area. The LI-6400 gas-exchange system also allowed the control of temperature (30 ± 0.2 °C), light intensity (610 μmol m^{-2} s^{-1}), relative humidity (40–50%) and CO_2 concentration (50–1500 ppm during A/C_i responses, otherwise fixed at 400 ppm) during measurements. The equations used to calculate photosynthesis were those previously derived by von Caemmerer & Farquhar (1981). Readings were taken from three to four leaves selected randomly from three trays, each day of dehydration and rehydration, between 3 and 4 h after dawn. Readings were taken over a time period of 5 min after reaching steady-state gas exchange, and the average of the technical repeats (at least three on three different leaves) for each measurement was calculated. A/C_i responses were analysed with the mechanistic model of CO_2 assimilation proposed by Farquhar, von Caemmerer & Berry (1980), and the relevant kinetic parameters, maximal velocity of carboxylation (Vc_{max}) and maximal electron transport rate (J_{max}) were estimated.

To measure VOC emissions, the outlet of the cuvette was disconnected from the LI-6400 system and the flow was

diverted into a silcosteel cartridge packed with 200 mg of Tenax (Agilent, Cernusco sul Naviglio, Italy). A volume of 8 L of air was pumped through the trap at a rate of 150 mL min^{-1}. The cartridge was analysed by gas chromatography-mass spectrometry (GC-MS). GC-MS analyses were performed with an Agilent 6850 gas chromatograph coupled to an Agilent 5975C Mass Selective Detector (Agilent Technologies, Wilmington, DE, USA) or with a Perkin Elmer Clarus 580 gas chromatograph coupled to a Perkin Elmer Clarus 560S Mass Selective Detector (Perkin Elmer, Inc., Waltham, MA, USA). The Agilent GC was supplied with a thermal desorber UNITY (Markes International Limited, Llantrisant, UK) whereas the Perkin Elmer GC was coupled to a thermal desorber TurboMatrix 300 (Perkin Elmer, Inc.). Both GCs were equipped with a splitless injector and a HP-5MS capillary column (30 m in length, 250 μm in diameter and 0.25 μm film thickness). The column oven temperature was kept at 40 °C for the first 5 min, then increased by 5 °C min^{-1} to 250 °C, and maintained at 250 °C for 2 min. Helium was used as carrier gas. The concentration of each volatile was calculated by comparison with the peak area of a gaseous standard. The GC-MS was calibrated weekly using cylinders with standard mixtures of the main isoprenoids generally emitted by plants (isoprene, α-pinene and limonene) at an average concentration of 60 ppb (Rivoira, Milan, Italy). Compounds were identified using the NIST library provided with the GC/MS ChemStation software on both Agilent and Perkin Elmer measurements. GC peak retention time was substantiated by analysis of parent ions and main fragments of the spectra.

Isoprene emission was also measured online, by diverting the air at the exit of the gas-exchange cuvette into a Proton Transfer Reaction-Mass Spectrometer (PTR-MS; Ionicon, Innsbruck, Austria), which allowed fast detection of isoprene. Once the exit of the gas exchange system had been connected to the PTR-MS and a steady state in isoprene emission had been reached, the cuvette containing the leaf was covered with a black cloth until the emission reached the lowest possible level (approximately 400 s), and the cloth was then removed again. The PTR-MS was operated in a single-ion mode to detect isoprene (protonated $m/z = 69$) down to a threshold of 1 ppt. Calibrations using an isoprene gaseous standard (60 ppt) were performed daily before measurements. Details on isoprene analysis by PTR-MS can be found in Tholl *et al*. (2006). VOC measurements were repeated at least six times on different leaves of different plants at each single RWC.

Statistical analyses

Means and standard errors were calculated with Graph Pad Prism (Version 5; GraphPad Software, Inc., La Jolla, CA, USA). Biological replications varied by experiment and are indicated in the appropriate figure legend. The significance of differences between means at different RWCs was analysed using Student's *t*-test.

RESULTS

The changes in photosynthesis during dehydration of *X. humilis* are shown in Fig. 1a. Net photosynthesis decreased rapidly below 80% RWC and had ceased by 57% RWC, when chlorophyll content had decreased about 50% (Fig. 1b). Recovery of photosynthesis was initiated upon rehydration above 60% RWC (Fig. 1a). This coincided with an increase in chlorophyll content (Fig. 1b) and the likely regeneration and reassembly of thylakoid membranes.

GC-MS analyses revealed that isoprene emission occurs from *X. humilis* plants. Isoprene emission was light dependent, as in all other isoprene emitters (data not shown) suggesting that emission is dependent on photosynthesis. Thus, the changes in emission of isoprene during dehydration and rehydration were assessed in relation to photosynthetic changes. There was a significant increase ($P < 0.001$) in isoprene emission, from 1 to more than 4 nmol m^{-2} s^{-1}, as

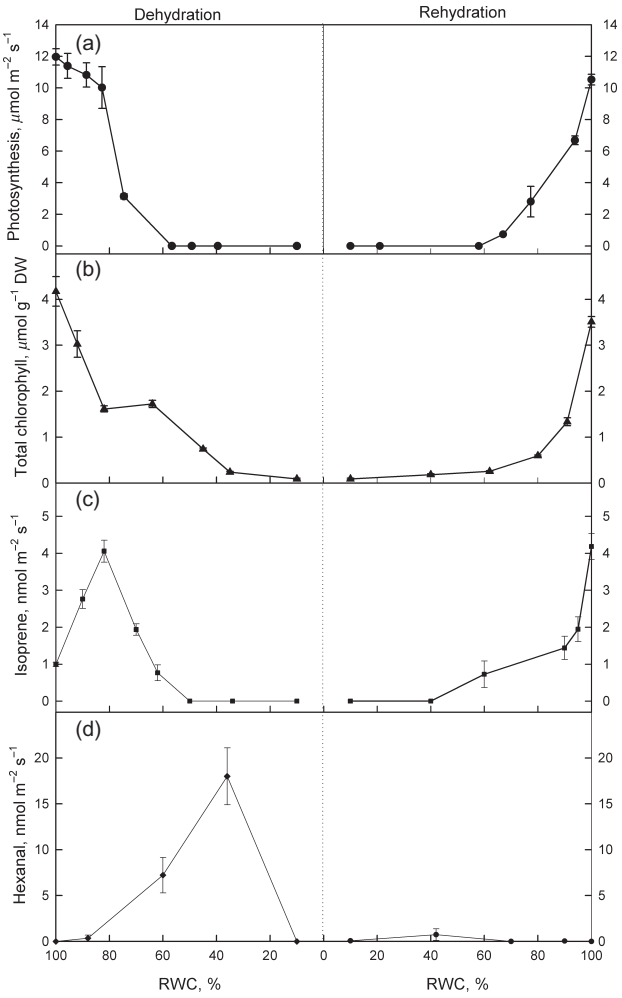

Figure 1. Change in photosynthesis (a) (mean ± SEM, $n = 5$), total chlorophyll content (b) (mean ± SEM, $n = 3$), isoprene emission (c) (mean ± SEM, $n = 6$), and hexanal (d) (mean ± SEM, $n = 3$) during dehydration (left panels) and rehydration (right panels) of *Xerophyta humilis* plants. DW, dry weight; RWC, relative water content.

the leaf RWC decreased from 100 to 82% (Fig. 1c). Isoprene emission then declined progressively until a RWC of 50%, after which no further emission was recorded. Photosynthesis in these leaves started to decline below a RWC of 80%, when isoprene emission was at its maximum, and had decreased once plant tissues had a recorded RWC of 62%. As the ratio of isoprene emission to photosynthesis increased in response to initial dehydration, the amount of carbon lost as isoprene increased exponentially as RWC decreased from 100 to 62%. At around 60% RWC, this ratio reached as much as 26% of the total carbon assimilated by photosynthesis (data not shown). During rehydration, photosynthesis recovered to prestress levels. However, isoprene emission recovered at a slower rate than photosynthesis. Upon full rehydration to 100% RWC, isoprene emission reached levels of 3.6–5.2 nmol m^{-2} s^{-1}, this being equivalent to the maximum emission upon dehydration, and much higher than it was prior to dehydration in *X. humilis*.

Analysis of the volatile samples collected during dehydration and rehydration using GC-MS revealed the emission of the oxygenated C_6 VOC hexanal (Fig. 1d). The emission of hexanal, which is a sensitive marker of membrane denaturation (Loreto *et al*. 2006; Capitani *et al*. 2009), increased in response to dehydration and peaked at 35% RWC, before decreasing rapidly during the very late stages of dehydration. During rehydration, there was no emission of hexanal detected from *X. humilis*, showing that lipoxygenase (LOX) activity is not stimulated during the recovery process.

The photosynthetic response to intercellular CO_2 (C_i) was already affected in leaves that had dehydrated to 85% RWC, but the effect was especially evident at CO_2 concentrations higher than ambient (Fig. 2). In rehydrated leaves, the photosynthetic response to C_i attained the prestress level indicating a complete recovery of photosynthesis and no permanent limitations caused by dehydration.

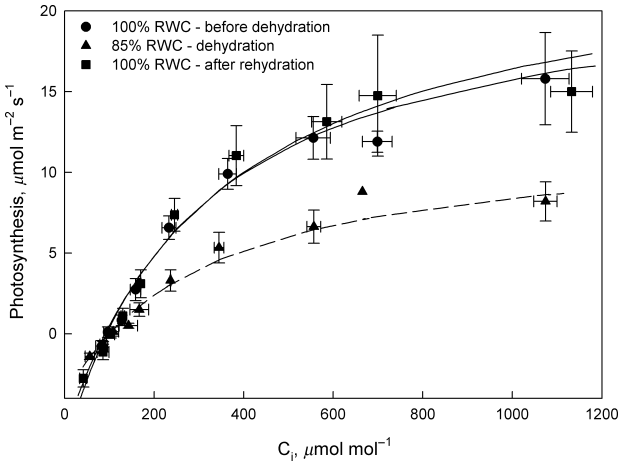

Figure 2. Response of photosynthesis to variations of intercellular CO_2, C_i, before dehydration [100% relative water content (RWC), circles], during early dehydration (85% RWC, triangles) and after rehydration (100% RWC, squares) of *Xerophyta humilis* plants (mean ± SEM, $n = 3$]). The curves were fitted using Farquhar, von Caemmerer & Berry (1980) model. Continuous lines fit the data points before dehydration and after rehydration at RWC = 100%; the dashed line fits data collected at RWC = 85%. The maximal electron transport rate (J_{max}) values calculated by Farquhar's model are shown in the text.

The change in chlorophyll fluorescence in leaves of *X. humilis* during dehydration is shown in Fig. 3a, with representative images of chlorophyll fluorescence taken at a whole plant level, and at 100, 60 and 25% RWC, respectively. On the side of each image, the actual average (± SEM) of the values for the given parameter is shown at various RWCs during dehydration. The initial fluorescence F_o, measured when the plant had been dark adapted and all reaction centres are assumed to be open, decreased slightly at the onset of dehydration but thereafter remained constant during further drying (Fig. 3a-i). The maximal fluorescence F_m, measured during the high-intensity rapid flash of light, which causes all reaction centres of darkened plants to close, is reached in the absence of photochemical and NPQ. As can be seen in the images of Fig. 3a-ii, F_m followed a similar trend to F_o during early stages of dehydration, but declined substantially also between 80 and 60% RWC.

The maximum efficiency of PSII in dark-adapted leaves (F_v/F_m) remained relatively high during the initial stages of dehydration and started to decline from about 80% until 60% RWC with a rapid decline from 20% RWC onwards (Fig. 3a-iii). As can be seen in the images for F_v/F_m, the false colour remained blue-purple even until 25% RWC, indicating at this stage that the efficiency of PSII, if all reaction centres were open, was still relatively high. The response curve in this study corresponds to that reported to date for resurrection plants.

Figure 3a-iv shows the actual quantum yield, for example, the true efficiency of photosystem II (Φ_{PSII}) during dehydration. Φ_{PSII} measures the rate of linear electron transport rate driving photosynthesis and photorespiration when leaves are illuminated and photosynthesis is activated. The images for Φ_{PSII} clearly show that the efficiency of PSII rapidly declined, and that the electron transport rate was very heterogeneous within each leaf during dehydration. The latter observation was quantitatively confirmed by separately assessing the fluorescence values measured in the basal third and in the apical third of the leaves (Table 1). Heterogeneities developed during dehydration in both maximal and actual quantum yield of fluorescence (F_v/F_m and Φ_{PSII}, respectively), being the apical part more sensitive to the stress. Because a lower electron transport rate produces photochemical limitations of photosynthesis, this indicated that the photochemical apparatus of the basal part of the leaves was more preserved from dehydration stress.

The NPQ of fluorescence, the mechanism whereby plants convert excess energy to heat and thus minimize subcellular damage especially in high light conditions, initially increased as the plants dehydrated from 100 to 80% RWC. However, this value declined below RWCs of 60% (Fig. 3a-v), this coinciding with the decrease in photosynthesis as chlorophyll was broken down. Moreover, no heterogeneity of NPQ of fluorescence was observed during the development of dehydration (Table 1).

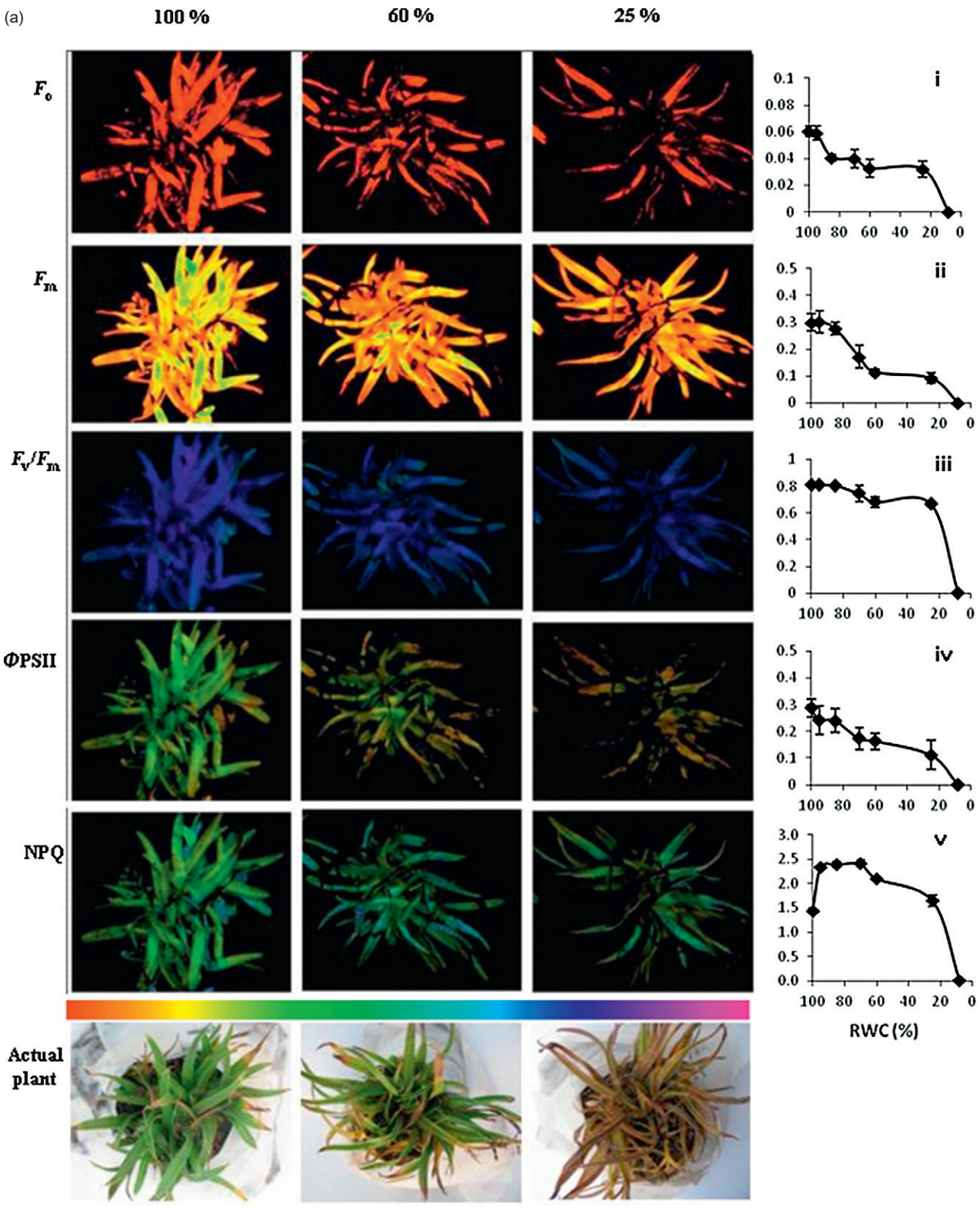

Figure 3. Chlorophyll fluorescence images of F_o, F_m and F_v/F_m in dark-adapted plants and of ΦPSII and non-photochemical quenching (NPQ) in *Xerophyta humilis* plants under a steady-state illumination of 610 μmol photons m^{-2} s^{-1}. In panel (a), images measured at 100, 60 and 25% relative water content (RWC) during dehydration are shown. In panel (b), the same fluorescence parameters are shown in rehydrating plants at 60, 85 and 100% RWC. In both panels, photographs of the actual plants at the correspondent RWC level are also shown at the bottom, and actual fluorescence parameter values (means ± SEM of $n = 7$ replications on different plants) are shown on the right column. The false colour code depicted ranges from 0 (black) to 1 (pink) in fluorescence intensity.

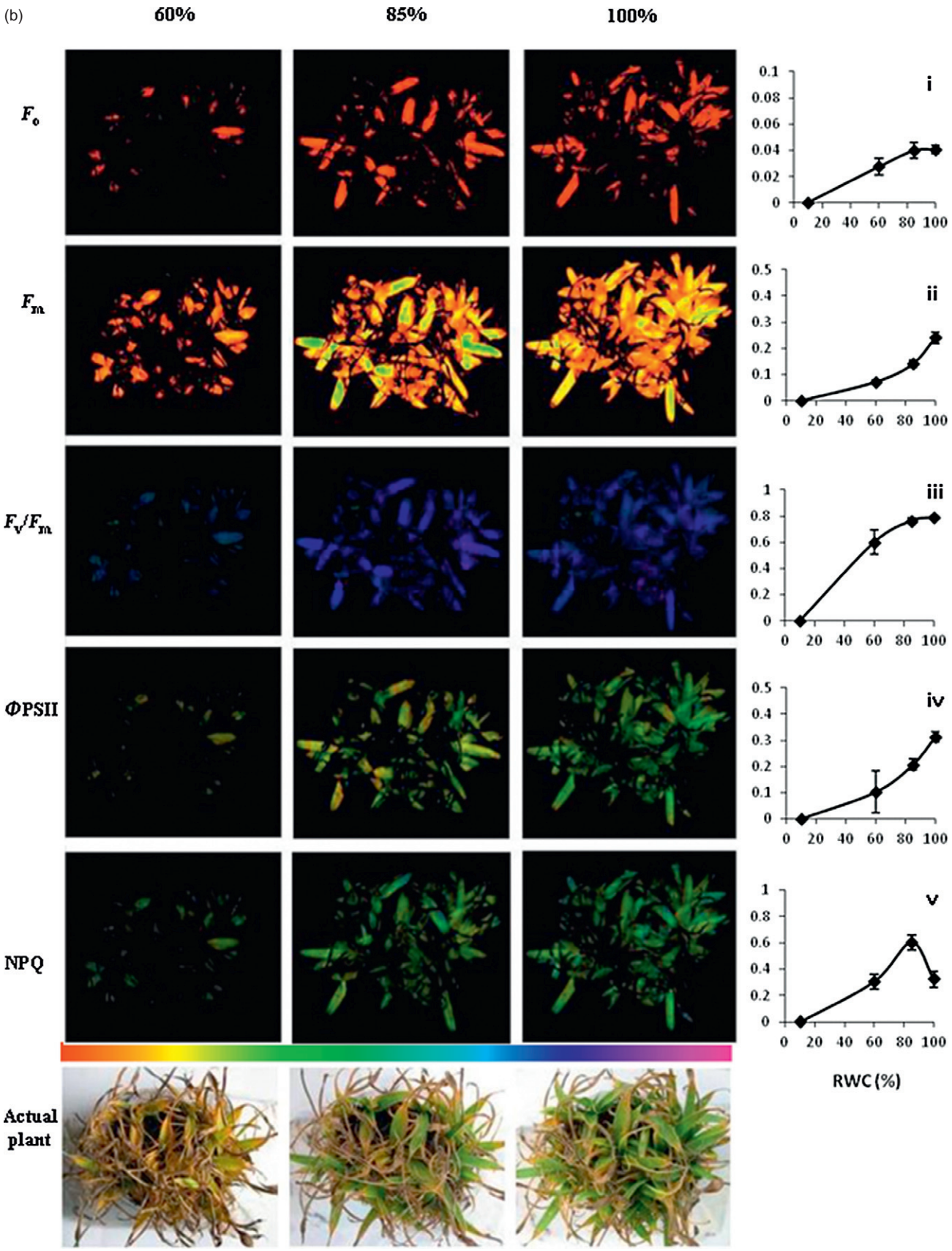

Figure 3. *Continued*

RWC	Leaf part	F_v/F_m	Φ_{PSII}	NPQ
Dehydration				
100%	Apical	0.754 ± 0.034^{a,b}	0.214 ± 0.012^{b}	1.96 ± 0.40^{a,b}
	Basal	0.791 ± 0.005^{a}	0.220 ± 0.050^{b,c}	2.21 ± 0.26^{a}
60%	Apical	0.685 ± 0.032^{c,d}	0.167 ± 0.004^{c}	1.70 ± 0.41^{a,b}
	Basal	0.748 ± 0.004^{b}	0.209 ± 0.002^{b}	1.88 ± 0.19^{a,b}
25%	Apical	0.633 ± 0.024^{d}	0.086 ± 0.002^{d}	1.30 ± 0.27^{b}
	Basal	0.705 ± 0.003^{c}	0.171 ± 0.010^{c}	1.53 ± 0.03^{b}
Rehydration				
60%	Apical	0.711 ± 0.022^{b,c}	0.139 ± 0.019^{c}	2.51 ± 0.29^{a}
	Basal	0.708 ± 0.006^{c}	0.230 ± 0.033^{b}	1.64 ± 0.07^{b}
85%	Apical	0.755 ± 0.013^{b}	0.159 ± 0.010^{c}	2.23 ± 0.23^{a}
	Basal	0.752 ± 0.014^{b}	0.219 ± 0.046^{bc}	1.59 ± 0.13^{b}
100%	Apical	0.749 ± 0.017^{b}	0.163 ± 0.005^{c}	2.15 ± 0.29^{a}
	Basal	0.801 ± 0.011^{a}	0.349 ± 0.011^{a}	1.56 ± 0.05^{b}

Table 1. Variations in fluorescence parameters within single leaves at different relative water contents (RWCs) during dehydration and rehydration of *Xerophyta humilis* plants

Maximal and actual quantum yield of fluorescence (F_v/F_m and Φ_{PSII}, respectively) and non-photochemical quenching of fluorescence (NPQ) were measured in the apical and basal parts of the leaves. Means ± SEM ($n = 4$) are shown. Leaves were put at the same distance from the fluorescence camera to avoid artefacts due to position. Differences of values of each parameter at varying RWC were assessed by Tukey's and are shown by different letters when significantly different at the 5% ($P < 0.05$)

During rehydration, there was full recovery of all chlorophyll fluorescence parameters, this process being initiated upon rehydration above 60% RWC, coincident with production of chlorophyll and initiation of photosynthesis, and being complete, with maximal fluorescence yield again achieved upon rehydration to 100% RWC (Fig. 3b). However, F_o and the maximal quantum yield of fluorescence appeared to fully recover already at 85% RWC, indicating that the photochemical apparatus was fully reconstituted at this rehydration stage (Fig. 3b-i and iii, respectively). On the other hand, the actual quantum yield of fluorescence (Φ_{PSII}, Fig. 3b-iv) only reached the maximum at 100% RWC and the NPQ (Fig. 3b-v) reached a maximum at 85% RWC but then dropped. These findings suggest that photochemical energy was safely dissipated as heat at 85% RWC whereas it became again used to produced photosynthetic electron transport rate only when the RWC recovery was complete, for example, that photosynthesis was still limited by processes that are not directly related to PSII structure at RWC = 85% during rehydration. Independent of the residual photosynthetic limitation during rehydration, the overall complete recovery (see also Figs 1–2) suggests that chloroplasts did not suffer from permanent damage upon dehydration, concurring previous reports for this and other *Xerophyta* species (Sherwin & Farrant 1998; Tuba *et al*. 1998; Collett *et al*. 2003; Ingle *et al*. 2008). Again, rehydration affected heterogeneously leaf fluorescence properties, the recovery being more rapid at the base of the leaves, whereas the leaf tips often did not rehydrate (Fig. 3b and Table 1).

During dehydration of *X. humilis*, there was a steady decline in violaxanthin from 0.13 μmol g^{-1} DW at 100% RWC to 0.005 μmol g^{-1} DW in dry leaf tissue (Fig. 4a). Zeaxanthin content increased significantly from 0.012 μmol g^{-1} DW in control leaves (100% RWC) to 0.082 μmol g^{-1} DW in the desiccated leaf tissue (10% RWC) (Fig. 4a).

During rehydration, the reverse reactions took place. As can be seen in Fig. 4a, zeaxanthin levels remained constant during the initial stages of rehydration and then decreased rapidly upon rehydration above 60% RWC, with violaxanthin increasing at this stage. At the end of rehydration, violaxanthin content was higher than it was prior to dehydration. Zeaxanthin remained high until 60% RWC.

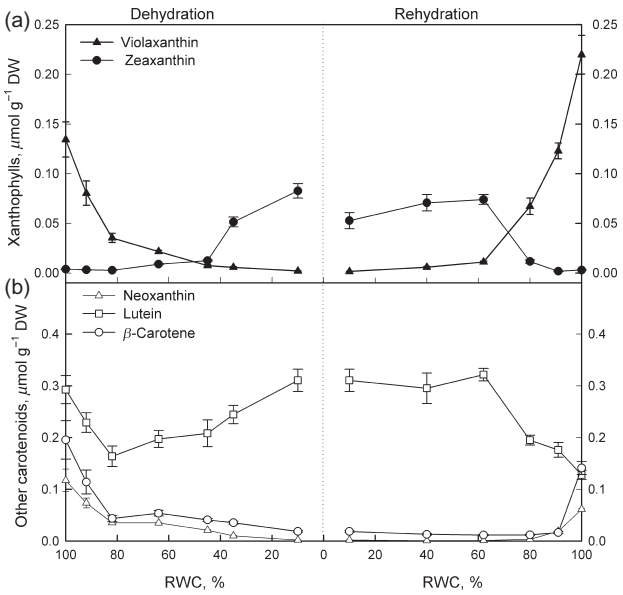

Figure 4. Change in zeaxanthin and violaxanthin (a) and other carotenoid pigments (b) during dehydration and rehydration of *Xerophyta humilis* plants. Values are calculated relative to dry weight (DW) (mean ± SEM, $n = 3$). RWC, relative water content.

Activation of the epoxidation of zeaxanthin back to violaxanthin thus seems to occur at 60% RWC during rehydration. Antheraxanthin levels did not change significantly.

The changes in the remaining carotenoids, which do not form part of the xanthophyll cycle, are shown in Fig. 4b. Neoxanthin decreased during dehydration, and upon rehydration it increased again and reached a level comparable to what it was before dehydration. The concentration of lutein initially decreased as the plants dehydrated from 0.292 μmol g^{-1} DW at 100% RWC to 0.164 μmol g^{-1} DW at 82% RWC, but increased during the latter stages of dehydration to levels slightly higher than the pre-desiccated condition. During dehydration, β-carotene progressively decreased.

GC-MS analyses revealed that isoprene emission occurs from *X. humilis* plants. Isoprene emission was light dependent, as in all other isoprene emitters (data not shown) suggesting that emission is dependent on photosynthesis. Thus, the changes in emission of isoprene during dehydration and rehydration were assessed in relation to photosynthetic changes in a set of measurements different from those shown in Fig. 1.

DISCUSSION

Regulation of photosynthesis

Photosynthesis in *X. humilis* was not particularly resistant to dehydration, and it started to be inhibited at relatively high RWCs. The response of photosynthesis to intercellular CO_2 concentration (A/C_i response) provides a rapid and reliable method to identify *in vivo* photosynthetic limitations (Farquhar & Sharkey 1982). Before introducing our discussion on A/C_i results, it should be said that, when stomata close in response to strong dehydration, an increasing contribution of (1) stomatal patchiness; (2) cuticular conductance, is often observed. This may produce an overestimation of C_i (Meyer & Genty 1998) and consequently impair the analysis of A/C_i responses. While the reader should be aware that this could also affect our A/C_i analysis, we observe that the impact of patchiness, as revealed also by our imaging fluorescence measurements, was minimized by selecting for gas-exchange measurements leaf areas that showed homogeneous fluorescence. We also reason that the impact of cuticular transpiration should be higher at low C_i (Meyer & Genty 1998), whereas in the present study, photosynthesis of dehydrated leaves was lower than in controls, especially at CO_2 concentrations higher than ambient, for example, when photosynthesis does not increase linearly with increasing C_i. This indicates photosynthesis limitations due to RuBP regeneration in plants undergoing mild dehydration (85% RWC). Because RuBP regeneration depends on efficient electron transport rate supplying sufficient reducing power (NADPH) and chemical energy (ATP), our data indicate a possible photochemical limitation of photosynthesis in mildly dehydrated *X. humilis* leaves. Indeed, the maximum electron transport rapacity (J_{max}) estimated after removing diffusive limitations according to Sharkey *et al.* (2007) from A/C_i best fitting (Fig. 2) dropped significantly from 90.5 $\pm$ 9.5 μmol m^{-2} s^{-1} before dehydration to 51.7 $\pm$ 4.6 μmol m^{-2} s^{-1} at 85% RWC. The possibility that light reactions limit photosynthesis in dehydrating leaves of *X. humilis* was further investigated by chlorophyll fluorescence analysis, as shown below. Interestingly, such a photochemical limitation developed simultaneously with the onset of chlorophyll degradation in dehydrating leaves. Thus, the reduction in photochemical activity might be due to chlorophyll degradation and/or, as has been proposed for homoiochlorophyllous resurrection plants, due to chlorophyll shading and masking by anthocyanins (Farrant 2000; Farrant *et al.* 2003). Chlorophyll had been entirely degraded by the time the plants desiccated, as has been reported in other studies on *Xerophyta* species (Tuba *et al.* 1996, 1998; Farrant 2000).

During rehydration, recovery of photosynthesis was initiated upon rehydration above 60% RWC (Fig. 1a). This coincided with the production of chlorophyll (Fig. 1b) and the regeneration and reassembly of thylakoid membranes. It has been proposed that this delay is required for other protection mechanisms, notably regeneration or activation of antioxidant activity in order to minimize the potential damage associated with photosynthetically produced ROS upon rehydration (Farrant 2007). Interestingly, the rehydrated leaves did not show any residual impact of the stress on the photosynthetic apparatus, as indicated by A/C_i responses of rehydrated leaves overlaying those obtained before dehydration. The calculated J_{max} after full rehydration was 89.6 $\pm$ 17.5 μmol m^{-2} s^{-1}, for example, very similar to that calculated in the same leaves before dehydration. This is interpreted to indicate total *de novo* synthesis of chlorophylls and to the consequent generation of a functional photochemical apparatus.

Chlorophyll fluorescence

The photochemical limitations of leaves of *X. humilis* during dehydration are clearly seen when using imaging chlorophyll fluorescence. Fluorescence analyses can give insights into a plant's capacity to withstand environmental stresses and also give a measure of the amount of damage a stress has caused to the photosynthetic apparatus (Maxwell & Johnson 2000). Imaging fluorescence adds to this capacity, the possibility to study spatial heterogeneities during stress development, for example, caused by leaf ontogeny or anatomy, or different sensitivity to stress.

Fluorescence analysis revealed that the maximum quantum yield of PSII (F_v/F_m), a specific indicator of photoinhibition (Maxwell & Johnson 2000), was not affected until considerable water loss (to less than 20% RWC) had occurred. Thus, photochemical limitations during the early phases of stress do not imply photo-inhibition. Rather, the rapid decrease of the minimum fluorescence (F_o) and of the photochemical quenching (qP) of fluorescence (data not shown, but mirroring Φ_{PSII} of which qP is the most important component, Genty *et al.* 1989) indicates that the number of photochemical reaction centres able to operate

basal fluorescence and photosynthetic electron transport (Genty *et al*. 1989) was somehow impaired already at the early stages of dehydration. Thus, it may be speculated that photochemical impairment in dehydrating leaves is due to the loss of active PSII centres rather to a reduced efficiency of their photochemical function. Imaging fluorescence clearly revealed that the fluorescence signal, hence the photochemical efficiency, was not homogeneous across the leaves. A main difference could be observed between the basal part of the leaves that continued to have a high photochemical efficiency until the tissues were dehydrated, and the apical parts in which such efficiency, and more specifically the actual quantum yield of PSII (Φ_{PSII}) which is indicative of the electron transport rate driving photosynthetic carbon reduction and carbon oxygenation cycles (Genty *et al*. 1989), was largely impaired during early stages of dehydration. Thus, it may be surmised that the inactive fraction of PSII in dehydrating leaves resides in the apical part of the leaves. Because apical tissues are older than that at basal regions, it is likely that the loss of PSII activity in the former is associated with the onset of senescence processes (Kikuzawa & Lechowicz 2011) possibly exacerbated by frequent cycles of drying and rehydration. This hypothesis is supported by the observation that often the leaf tips of monocot resurrection plants, and older leaves of dicot species, do not rehydrate (Farrant 2000, 2007; Vander Willigen *et al*. 2001).

During rehydration, there was full recovery of all chlorophyll fluorescence parameters. Such recovery correlates with the results from A/C_i response curves and suggests that chloroplasts became fully functional upon rehydration, concurring with previous reported for this and other *Xerophyta* species (Tuba *et al*. 1993a, b; Sherwin & Farrant 1998; Ingle *et al*. 2008). While NPQ followed the same initial trend, with values increasing to 2.4 upon rehydration to 85% RWC, full rehydration resulted in a subsequent decline of this parameter to values equivalent of those prior to dehydration (compare Fig. 3a-v and 3b-v). These data are of interest, as they indicate the capacity to rapidly and efficiently regulate the dissipation of excess light non-radiatively in this resurrection plant. They also suggest that at 85% RWC, despite PSII not being fully operational, sufficient protection had accrued in rehydrating tissues such that dissipation of excess energy as heat could be reduced.

Pigment analyses

Carotenoids play a significant role in photoprotection as they can quench ROS, thereby reducing permanent damage associated with excess excitation energy under stress situations including drought (Demmig-Adams & Adams III 1996; Munné-Bosch & Alegre 2000). There was a steady decline in violaxanthin from the start of dehydration and a considerable increase in zeaxanthin content once the RWC had declined below 40%. The reverse was observed in rehydrating leaves, but interestingly, zeaxanthin did not revert into violaxanthin until the leaf RWC was greater than 60%. These changes are likely to be due to the well-known pathway of de-epoxidation of violaxanthin into zeaxanthin via the intermediate antheraxanthin, with concomitant dissipation of excess solar radiation heat, a phenomenon reported as 'the xanthophyll cycle', with resulting antioxidant action and photoprotection (Demmig *et al*. 1988; Demmig-Adams & Adams III 1996). This mechanism is generally activated by dark-to-light transitions, but here we show that the same mechanism may be induced by dehydration in *X. humilis*. Although *X. humilis* is poikilochlorophyllous, photosynthetic-generated ROS are likely to occur during the early stages of dehydration when chlorophyll has not been completely degraded. However, changes in xanthophylls de-epoxidation only occurred when plants were severely dehydrated and there was no physiological activity. Thus, it is unlikely that in *X. humilis,* the xanthophyll cycle serves primarily to quench ROS formation during dehydration. The increase in zeaxanthin biosynthesis paralleled the considerable reductions in chlorophyll concentration, and presumably originated by the action of violaxanthin de-epoxidase and/or β-carotene hydroxylase. β-carotene hydroxylase synthesizes zeaxanthin from β-carotene. Increased zeaxanthin was found to confer drought tolerance in Arabidopsis (Davison *et al*. 2002) and rice plants (independent of zea-induced ABA biosynthesis, Du *et al*. 2010). Zeaxanthin concentration accounted for 185 mmol mol^{-1} total chlorophyll at 35% RWC, and increased steeply to reach 650 mmol mol^{-1} total chlorophyll at 10% RWC. This high concentration in zeaxanthin is unlikely to be bound to light-harvesting chlorophyll-protein complexes (Havaux & Niyogi 1999). Zeaxanthin may therefore reside in other parts of the thylakoids. Zeaxanthin, but not β-carotene, may increase rigidity of thylakoid membranes, and reduce peroxidative damage under drought stress (Havaux *et al*. 1996, 2007), and we surmise that this is the predominant role of zeaxanthin in this resurrection plant, in coordination with isoprene biosynthesis and emission (see below). This confirms that photosynthetic membranes, while dismantled (Sherwin & Farrant 1998; Farrant 2000), are not completely destroyed in dry leaves of *X. humilis* (Dace *et al*. 1998; Ingle *et al*. 2008).

Among the remaining carotenoids, the changes in lutein seem particularly interesting. Indeed, the concentration of lutein rapidly decreased, but then increased again during the latter stages of dehydration, to levels slightly higher than the pre-desiccated condition. The sequence of lutein stimulation is similar to that observed for zeaxanthin and is likely to reflect a similar function of these compounds under dehydration. Lutein is also suggested to reduce oxidative damage during dehydration (Demmig-Adams & Adams III 2002) and plays an important role in NPQ quenching (Niyogi, Björkman & Grossman 1997). In addition to this potential role, we propose that lutein, like zeaxanthin, might act as a membrane strengthener during dehydration in *X. humilis*.

Contrary to lutein, β-carotene concentration progressively decreased during dehydration. It is probable that the decline in β-carotene was due to its expenditure as an antioxidant and/or its absorption of excess light and therefore

protecting the plant from oxidative damage (Telfer 2002). β-carotene reduction might also occur if the same pool of carbon that generates isoprenoids is temporarily allocated to more effective defensive molecules such as zeaxanthin, lutein and isoprene (see below). β-carotene was re-synthesized again only during the late stages of rehydration possibly effecting some protection as photosynthesis is once again initiated. However, because it is also an accessory pigment to photosynthesis, it is likely to be involved in the induction of photosynthesis occurring at this stage. These trends in β-carotene are comparable with those observed in other resurrection plants (Kranner *et al*. 2002).

Volatile emissions

Isoprene, one of the most abundant biogenic VOCs (Loreto & Schnitzler 2010), is mainly emitted by hydrophilic plants (Vickers *et al*. 2009). This is the first report that isoprene is possibly emitted by a resurrection plant that is adapted to recurrent dehydration events. The discovery that isoprene emission may be occurring in such plant species has an important ecological value.

In light of the hypothesized roles of isoprene in providing protection against lipid peroxidation and oxidative damage and in facilitating membrane stabilization during abiotic stress (Loreto *et al*. 2001; Sharkey & Yeh 2001; Velikova, Edreva & Loreto 2004; Peñuelas & Munné-Bosch 2005; Vickers *et al*. 2009; Velikova *et al*. 2011), it is possible that isoprene also contributes to the protection mechanisms during desiccation of *X. humilis*. Indeed, more than 20% of the photosynthetic carbon is allocated into isoprene under severe dehydration, suggesting an important function of this molecule in dehydrating leaves. As in previous studies on desiccation-sensitive plants, which have been subjected to a drought stress (Loreto & Sharkey 1993; Fang, Monson & Cowling 1996; Pegoraro *et al*. 2004; Brilli *et al*. 2007), isoprene emission is firstly stimulated and then responds more slowly in time to drought than photosynthesis. The reason for the transient increase in isoprene emission during drought stress is still not known. Among the most likely explanations are that: (1) transiently higher foliar temperatures in plants recovering from stress, possibly in turn due to lower latent heat dissipation due to stomatal closure. The temperature dependence of isoprene emission is a well-reported phenomenon (Loreto & Sharkey 1990; Niinemets, Loreto & Reichstgein 2004); or (2) lower intercellular CO_2 concentration, again a consequence of drought-induced stomatal closure. Low intercellular CO_2 has been recently been reported to positively affect isoprene emission in a range of cultivated poplars (Guidolotti, Calfapietra & Loreto 2011).

During rehydration of *X. humilis*, isoprene emission recovered at a slower rate than photosynthesis. This could be related to the differences in severity of water deficit experienced (95% of total cellular water) prior to recovery by resurrection plants compared with the relatively mild water deficits imposed on desiccation-sensitive plants in similar experiments. Recovery of photosynthesis is delayed in poikilochlorophyllous resurrection plants, as re-establishment of this metabolism requires re-synthesis of chlorophyll and reassembly of thylakoid membranes (Farrant 2000; Ingle *et al*. 2008). We propose the slow recovery of isoprene emission, and the absence of the typical post-drought burst of emission, might be consequences of the need to reassemble the photosynthetic machinery before carbon could be assimilated for the production of isoprene in *X. humilis*.

Isoprene drops to values below those measured in fully hydrated leaves at the same RWC at which zeaxanthin biosynthesis started to be stimulated during dehydration. Similarly, isoprene is again emitted when zeaxanthin and lutein biosynthesis drop in rehydrating leaves. The inverse temporal correlation between isoprene emission and zeaxanthin concentration in our experiment appears of particular interest, as both isoprenoids may confer thermo-tolerance to thylakoid membranes (Havaux *et al*. 1996; Loreto & Schnitzler 2010). Isoprene might function in early stages of dehydration, when the electron transport rate can still drive photosynthesis and photosynthesis-dependent carbon fixation into volatile isoprenoids, whereas zeaxanthin might preserve thylakoid membranes from more severe oxidative damage/disruption (Havaux *et al*. 2007). This well-coordinated mechanism of membrane defence against dehydration damage, driven by non-volatile and volatile isoprenoids, may be an integral part of the resistance and revival mechanism in poikilochlorophyllous resurrection plants.

In addition to isoprene, VOC analysis revealed the emission of the oxygenated C_6 VOC hexanal during dehydration (Fig. 1d). The biochemical pathway leading to the formation of this and other oxygenated VOCs has been well documented (Croft, Juttner & Slusarenko 1993; Hatanaka 1993). LOXs catalyse the addition of oxygen to polyunsaturated fatty acids to produce an unsaturated fatty acid hydroperoxide. In plants, the substrates for LOX are linoleic and linolenic acid, which are common constituents of the plant membranes (Croft *et al*. 1993). LOX enzymes are reported to preferentially act on free fatty acids, which are generated from cell membranes in response to ROS accumulation under stressful conditions (Porta & Rocha-Sosa 2002; Beauchamp *et al*. 2005). LOX activity produces 9- or 13-hydroperoxylinoleic or -linolenic acid, or a mixture, and the degradation of the hydroperoxides leads to the formation of volatile C_6 compounds (Heiden *et al*. 2003).

As can be seen in Fig. 1d, the emission of hexanal increased in response to dehydration and peaked at 35% RWC, before decreasing rapidly during the very late stages of dehydration. LOX-generated C_6-oxygenated VOCs may be used as a proxy of membrane denaturation (Capitani *et al*. 2009). Thus, confirming previous reports of thylakoid disassembly into membranous vesicles (Farrant 2000), it may be suggested that this process started at around 35% RWC during dehydration of *X. humilis* leaves. Because no emission of hexenal was observed in rehydrating leaves, it may be inferred that rehydration does not affect further membrane structure, or that the capacity to emit

C_6-oxygenated VOCs has ceased. A study conducted in clover also found that the aldehyde (Z)-3-hexenal and the alcohol (Z)-3-hexenol were produced during the drying process (De Gouw *et al*. 1999) and LOX activity has been monitored in olive trees and shown to increase during the progression of water deficit (Sofo *et al*. 2004). Those authors proposed that production of LOX-generated C_6 VOCs could serve as secondary messengers serving *inter alia* as transcription factors that in turn activate drought stress-associated genes.

As for volatile and non-volatile isoprenoids, the sequence at which different compounds related to membrane integrity appear is of particular interest. Hexanal emission only increased once isoprene emission started to decrease, peaking when isoprene emission had been completely inhibited and zeaxanthin is synthesized at the highest rate in dehydrating leaves. One of the proposed roles of isoprene is that, as a small lipophilic molecule, it might enhance hydrophobic interactions within membranes or protein complexes (Singsaas *et al*. 1997; Sharkey & Yeh 2001). Indeed, there is accumulating evidence for the hypothesized role of stabilizing chloroplast membranes, especially during high temperature and ozone stress (Velikova & Loreto 2005; Velikova, Fares & Loreto 2008). It could be hypothesized that, in *X. humilis*, C_6 LOX accumulation and emission at RWC lower than 60% reveal damage to membrane structures that are specifically protected by isoprene, for example, the ordered arrays of light-harvesting complex PSII in the stacked region of the thylakoid grana (Velikova *et al*. 2011). However, when plants are severely dehydrated, membrane properties associated with isoprene protection appear not to be present and nor is there the release this class of volatile compound.

CONCLUSIONS

We have shown that photosynthesis is limited by light-dependent RuBP regeneration during early dehydration stages in the poikilochlorophyllous resurrection plant *X. humilis*. To cope with light stresses, all photosynthesizing organisms make use of conserved mechanisms of photoprotection. To reduce photo-oxidative damage during dehydration, resurrection plants may operate alternative, non-ubiquitous strategies. Among these, isoprene may have an important role for protection of photosynthetic membranes during early stages of dehydration, possibly in cooperation with other non-volatile isoprenoids.

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