

RESEARCH PAPER

# Restoration of photosystem II photochemistry and carbon assimilation and related changes in chlorophyll and protein contents during the rehydration of desiccated *Xerophyta scabrida* leaves

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## Abstract

**Recovery of photosynthesis in rehydrating desiccated leaves of the poikilochlorophyllous desiccation-tolerant plant *Xerophyta scabrida* was investigated. Detached leaves were remoistened under 12 h light/dark cycles for 96 h. Water, chlorophyll (Chl), and protein contents, Chl fluorescence, photosynthesis–CO<sub>2</sub> concentration response, and the amount and activity of Rubisco were measured at intervals during the rehydration period. Leaf relative water contents reached 87% in 12 h and full turgor in 96 h. Chl synthesis was slower before than after 24 h, and Chl<sub>a</sub>:Chl<sub>b</sub> ratios changed from 0.13 to 2.6 in 48 h. The maximum quantum efficiency recovered faster during rehydration than the photosystem II operating efficiency and the efficiency factor, which is known to depend mainly on the use of the electron transport chain products. From 24 h to 96 h of rehydration, net carbon fixation was Rubisco limited, rather than electron transport limited. Total Rubisco activity increased during rehydration more than the Rubisco protein content. Desiccated leaves contained, in a close to functional state, more than half the amount of the Rubisco protein present in rehydrated leaves. The results suggest that in *X. scabrida* leaves Rubisco adopts a special, protective conformation and recovers its activity during rehydration through modifications in redox status.**

**Key words:** Chlorophyll fluorescence, desiccation tolerance, non-photochemical quenching, photosynthesis, poikilochlorophyll, relative water content, Rubisco, *Xerophyta scabrida*.

## Introduction

Desiccation-tolerant (DT) plants can withstand the loss of up to 90–95% of the water of their vegetative tissues and revive when humidity is available, in contrast to the majority of plants (Proctor and Tuba, 2002). Desiccation tolerance entails cellular, biochemical, and molecular changes during dehydration (Vicré *et al.*, 2004), including the accumulation of carbohydrates (Whittaker *et al.*, 2001;

Toldi *et al.*, 2009), late embryogenesis-abundant (LEA) proteins (Ingram and Bartels, 1996), and antioxidants (Kranter *et al.*, 2002; Mowla *et al.*, 2002; Vicré *et al.*, 2004), as well as altered expression of target genes and transcription factors (Frank *et al.*, 1998; Ramanjulu and Bartels, 2002). Recovery from the desiccated state is much faster in homoiochlorophyllous DT (HDT) plants such as

Abbreviations: Chl, chlorophyll; DT, desiccation tolerant; HDT, homoiochlorophyllous DT; PDT, poikilochlorophyllous DT; RuBP, ribulose-1,5-bisphosphate; RWC, relative water content.

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*Haberlea rhodopensis* (Georgieva *et al.*, 2005, 2007) than in poikilochlorophyllous DT (PDT) plants such as *Xerophyta scabrada* (Tuba *et al.*, 1993a, 1994). The former retain their chlorophyll (Chl), preserve their photosynthetic apparatus, and undergo morphological changes during drying that protect their tissues against oxidative stress (Vicré *et al.*, 2004). In contrast, the latter lose all of their Chl and dismantle their photosynthetic apparatus during drying, and they resynthesize these molecules after rehydration (Tuba *et al.*, 1994, 1998a; Sherwin and Farrant, 1996). *Xerophyta scabrada* preserves most of its Chl when dried in the dark, so most of the loss seems to result from photooxidative degradation (Tuba *et al.*, 1997). The PDT strategy evolved in plants that are anatomically complex and that include the largest in size of all DT species, and it can be seen as the younger strategy in evolutionary terms (Proctor and Tuba, 2002).

DT plants regain their water content within a time span ranging from minutes in bryophytes and pteridophytes (Csintalan *et al.*, 1999) to days in angiosperms (Tuba *et al.*, 1994; Proctor and Tuba, 2002; Georgieva *et al.*, 2005; Degl'Innocenti *et al.*, 2008). As could be expected for HDT plants, the Chl contents, the Chl $a$ :Chl $b$  ratio, and the relative amounts of the Chl–protein complexes remain mostly unchanged in control, desiccated, and rehydrated leaves (Georgieva *et al.*, 2005, 2007). In remoistened PDT plants, Chl resynthesis begins after 6–12 h of rehydration (Tuba *et al.*, 1993a, 1994) at 36% relative water content (RWC) (Degl'Innocenti *et al.*, 2008), and is completed by 48–72 h at 84% RWC. Synthesis of the photosystem II (PSII) reaction centre and antenna proteins correlates with the recovery and increase in photosynthetic capacity (Ingle *et al.*, 2008).

Non-radiative energy dissipation can play an important protective role during both desiccation and rehydration. In several mosses (Csintalan *et al.*, 1999) and in *Ramonda serbica* (Augusti *et al.*, 2001; Degl'Innocenti *et al.*, 2008), non-photochemical quenching (NPQ) shows a transient increase upon remoistening. Maximum quantum efficiency,  $F_v/F_m$ , is completely recovered at 48 h (Degl'Innocenti *et al.*, 2008) or 72 h (Tuba *et al.*, 1994) after rewetting. Faster increases during rehydration were recorded in  $F_v/F_m$  than in PSII operating efficiency,  $F_q'/F_m'$  (also termed  $\Phi_{PSII}$ ; Csintalan *et al.*, 1999). Nonetheless, the involvement in the non-photochemical energy dissipation of basal, non-radiative decays and of the regulated non-photochemical energy loss (Baker *et al.*, 2007; Klughammer and Schreiber, 2008) during the rehydration of PDT plants has not been investigated.

Full recovery of photochemical activity in PDT plants requires the assimilation of CO $_2$  as an acceptor of the products of photosynthetic electron transport. In the moss *Polytrichum formosum*, carbon fixation is completely restored 3 h after rewetting (Proctor *et al.*, 2007) but is resumed at 12 h (51% RWC), and is not fully re-established at 48 h (84% RWC) after rehydration in higher plant DT species (Tuba *et al.*, 1994; Degl'Innocenti *et al.*, 2008). Recently, photosynthesis–CO $_2$  concentration responses in

hydrated HDT leaves were reported (Peeva and Cornic, 2009), but information concerning the fate of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) and the relative capacities of Rubisco carboxylation and electron transport in rehydrating PDT plants is scarce. In earlier studies, desiccated fronds (Harten and Eickmeier, 1986) and leaves (Daniel and Gaff, 1980) of DT plants conserved from 40% to 62% of the control Rubisco activity. A decrease in Rubisco content was observed during dehydration of the C $_4$  DT plant *Sporobolus stapfianus* (Martinelli *et al.*, 2007), whereas Rubisco (fraction I) protein did not appear to decrease relative to hydrated *X. viscosa* leaves (Daniel and Gaff, 1980). Consistent with this, it was surmised that the carboxylating enzymes in *X. scabrada* would only be inactivated, but not degraded, during desiccation (Tuba *et al.*, 1998b). In contrast, Rubisco activity was undetectable below 51% RWC (12 h rehydration) in *R. serbica* leaves (Degl'Innocenti *et al.*, 2008). Drying-induced disruption of the electron transport chain causes oxidative stress (Vicré *et al.*, 2004), which can induce aggregation and polymerization, membrane association, and the degradation of Rubisco (Marín-Navarro and Moreno, 2006). On the other hand, in stressed *Lemna minor* fronds Rubisco was not degraded but gradually became polymerized to inactive aggregates, accompanied by a reduction in the number of sulphhydryl groups (Ferreira and Shaw, 1989). The Benson–Calvin cycle enzymes have a tendency to form soluble and membrane-bound multienzyme complexes (Sainis and Harris, 1986; Gontero *et al.*, 1988, 1993; Sainis *et al.*, 1989; Persson and Johansson, 1989; Hermoso *et al.*, 1992; Anderson *et al.*, 1995; Agarwal *et al.*, 2009) with higher catalytic efficiency and less susceptibility to auto-oxidation and proteolysis than free enzymes (Gontero *et al.*, 1988, 1993).

The aim of this study was to determine to what extent the recovery from desiccation of *X. scabrada* photosynthesis is dependent on photochemical and carboxylation capacities. The hypothesis was that restoration of Rubisco activity limits the attainment of photosynthetic competence of rehydrated PDT plants. To test this hypothesis, Chl fluorescence and photosynthesis–CO $_2$  response curves were determined while turgor was being regained. To assess the carboxylation capacity, the free or aggregated state, as well as the amount and activity of Rubisco were determined in desiccated and rehydrating leaves.

## Materials and methods

A description of *X. scabrada* morphology has been provided in an earlier article (Tuba *et al.*, 1993b). Briefly, it is a 40–90 cm high, branched pseudoshrub with perennial leaves. Dry leaves are usually 24–30 cm long, 5–6 mm wide, and folded over along the midrib. In July 2004, desiccated *X. scabrada* (Pax) Th. Dur. et Schinz branches were collected in Tanzania (Mindu Hill, WSW of Morogoro town, 6°50.78'S, 37°36.76'E) and were kept in paper bags at room temperature until rehydration and analysis. Central sections of desiccated leaves having a purple-black or blackish-green coloration were selected for this study. As representative of time zero immediately prior to watering, triplicate desiccated leaves were briefly immersed in water in a vacuum desiccator to saturate

the intercellular air spaces with water. Subsequently the leaf material was blotted, weighed, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  for Chl, protein, and Rubisco activity measurements (see below). Previous experience (Tuba *et al.*, 1993b) has shown that placing whole plants with their roots in water does not result in a recovery response in *X. scabrida*, because the roots are dry and unable to transport water to the leaves; only a direct rewatering of the leaves by immersion in water led to regreening. Moreover, in the natural habitat, new root development and water uptake were preceded by the rehydration and regreening of the leaves. Consequently, in the present experiments, additional leaf samples were rehydrated by submerging them in a 10.0 l glass tank filled with tap water and aerated with a pump (Tuba *et al.*, 1993a, 1994). The container was placed in a growth chamber with a  $21^{\circ}\text{C}/15^{\circ}\text{C}$  day/night temperature, under a  $340\ \mu\text{mol m}^{-2}\text{ s}^{-1}$  photon flux density in a 12 h photoperiod (modified after Tuba *et al.*, 1994). The water was changed daily.

#### Water contents

Triplicate samples of desiccated leaves were weighed before and after drying in an oven for 48 h at  $60^{\circ}\text{C}$ ; the second of these recordings was taken as the dry weight. This was preferred to the oven-dry weight after full rehydration, which may be affected by losses during rewatering due to respiration or release of soluble compounds. Additional leaves (in triplicate) that were submerged in water were blotted and their fresh weight was determined at 12, 24, 48, 72, 96, and 120 h after the start of rehydration. No further weight gain was recorded after 96 h and this was considered as the turgid weight. The RWCs at successive times in the rehydration period were determined as  $(\text{fresh weight} - \text{dry weight}) \times 100 / (\text{turgid weight} - \text{dry weight})$ . Chl and protein contents, and Rubisco activity were determined in other leaves sequentially sampled during rehydration (see below) and were expressed on a turgid weight basis. The latter was estimated from the fresh weight of these leaf samples and the water contents measured in the samples used for RWC measurements.

#### Chl fluorescence and gas exchange measurements

For Chl fluorescence and  $\text{CO}_2$  assimilation measurements, triplicate leaf samples kept in water were collected between 3 h and 8 h after the start of the photoperiod at the times indicated above and placed in the fluorometer leaf clip or the infrared gas analyser (IRGA) leaf chamber (see below) with both ends of the leaves wrapped in moistened filter paper. After measurements, the leaf samples were returned to the water container in the growth room for a 30 min adaptation period prior to harvesting for leaf analyses (see below). Chl fluorescence was measured with a modulated fluorometer (PAM-2000, Walz, Effeltrich, Germany). Leaf sections were kept in the dark for 20 min with leaf clips (Gutiérrez *et al.*, 2009), after which dark-adapted state fluorescence parameters were measured.  $F_0$  was recorded and a saturating flash of light ( $\sim 8000\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ ) was applied for 0.8 s to determine  $F_m$ .  $F_0$  and  $F_m$ , respectively, represent the minimal and maximal fluorescence in the dark-adapted state, and  $F_v/F_m [(F_m - F_0)/F_m]$  represents the maximum quantum efficiency. Light-adapted leaves were illuminated with the red actinic light source of the fluorometer to obtain an irradiance of  $1500\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ . Saturating light pulses were given every 20 s until steady-state Chl fluorescence parameter values were obtained, the fluorescence values being recorded immediately before ( $F'$ , steady-state fluorescence) and after ( $F_m'$ , maximal fluorescence in the light) each pulse. Then, the leaf was covered with a black cloth, the actinic light was switched off, and an infrared light was switched on for 3 s to quickly reoxidize the PSII centres and measure  $F_0'$ , the minimal fluorescence with an NPQ similar to that found in the steady-state under light. The equipment determines  $F_q'/F_m' [(F_m' - F')/F_m']$ , which is the PSII operating efficiency (also termed  $\Phi_{\text{PSII}}$ ) (Baker *et al.*, 2007). The PSII efficiency factor  $F_q'/F_v'$  (also termed  $qP$ )  $[(F_m' - F')/(F_m' - F_0')]$

and  $F_v'/F_m' [(F_m' - F_0')/F_m']$ , the PSII maximum efficiency under light, were calculated. The fraction of PSII centres in the open state,  $qL$ , equates to  $(F_q'/F_v') (F_0'/F')$ . The quantum yield of basal, non-radiative decays,  $\Phi_{\text{NO}}$ , is  $1/[\text{NPQ} + 1 + qL(F_m'/F_0 - 1)]$ , where NPQ is  $(F_m'/F_m') - 1$ , and the quantum yield of non-photochemical quenching,  $\Phi_{\text{NPQ}}$ , is  $1 - (F_q'/F_m') - \Phi_{\text{NO}}$  (Kramer *et al.*, 2004).

Light-saturated photosynthesis- $\text{CO}_2$  response curves of leaves were recorded at the same times and with the same sampling scheme as Chl fluorescence. Measurements were carried out with an air flow rate of  $300\ \text{ml min}^{-1}$ ,  $1500\ \mu\text{mol m}^{-2}\text{ s}^{-1}$  irradiance, and a  $1.6 \pm 0.23\ \text{kPa}$  vapour pressure deficit, using a  $1.7\ \text{cm}^2$  window leaf chamber connected to a portable IRGA (CIRAS-2, PP Systems, Hitchin, Herts, UK) with differential operation in an open system. Temperature was kept at  $25^{\circ}\text{C}$  with the Peltier system of the IRGA. The air  $\text{CO}_2$  concentration was decreased in four steps from 34 Pa to 6 Pa and then increased from 34 Pa to 180 Pa in six steps. Chloroplast  $\text{CO}_2$  concentration,  $C_c$ , the maximum carboxylation rate allowed by Rubisco,  $V_{\text{cmax}}$ , and the rate of photosynthetic electron transport,  $J$ , were determined from the photosynthesis responses to  $\text{CO}_2$  with the Rubisco kinetic parameters and the Excel utility of Sharkey *et al.* (2007).

#### Rubisco activity assay

Triplicate leaf samples that had been equilibrated in aerated water in the growth chamber after Chl fluorescence and gas exchange measurements were blotted dry, rapidly transferred *in situ* to liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until analysed. Rubisco activity was assayed on the basis of the procedure described by Lilley and Walker (1974), modified by Ward and Keys (1989) and Sharkey *et al.* (1991). Aliquots (80 mg) of frozen leaves were ground in a mortar with liquid nitrogen, extracted with 4 ml of 100 mM *N,N*-bis(2-hydroxyethyl)glycine (Bicine)-NaOH (pH 7.8), 10 mM  $\text{MgCl}_2$ , 0.5 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1% (v/v) Triton X-100, 0.25% (w/v) bovine serum albumin (BSA), 20% (v/v) glycerol, 1 mM benzamidine, 1 mM  $\epsilon$ -aminocaproic acid, 10  $\mu\text{M}$  leupeptin, and 1 mM phenylmethylsulphonyl fluoride (PMSF), and then centrifuged at 13 000 g. The total time from extraction to the assay of initial Rubisco activity was  $< 2.5$  min. Activity was assayed by adding extract (40  $\mu\text{l}$ ) to a mixture of 100 mM Bicine (pH 8.2), 20 mM  $\text{MgCl}_2$ , 10 mM  $\text{NaHCO}_3$ , 18 mM KCl, 0.6 mM ribulose-1,5-bisphosphate (RuBP), 0.2 mM NADH, 1 mM ATP, 5 mM creatine phosphate, 25  $\text{U ml}^{-1}$  phosphocreatine kinase, 47  $\text{U ml}^{-1}$  phosphoglycerate kinase, 47  $\text{U ml}^{-1}$  glyceraldehyde 3-phosphate dehydrogenase, 10 mM DTT, 1 mM EDTA, 0.02% (w/v) BSA (800  $\mu\text{l}$  total volume) and recording the decrease in absorbance at 340 nm minus 400 nm for 40–60 s, at a stoichiometry of 2:1 between NADH oxidation and RuBP carboxylation. The spectrophotometer cell compartment was thermostated with a circulating water bath. To assay total Rubisco activity, an aliquot of the extract was incubated with  $\text{NaHCO}_3$  and  $\text{MgCl}_2$  for 10 min at room temperature before the addition of coupling enzymes and NADH; the reaction was started by adding RuBP. The activation state was estimated as initial activity, as a percentage of total activity. Activation and assays were performed either at room temperature or at  $35^{\circ}\text{C}$ . Commercial coupling enzymes suspended in ammonium sulphate were precipitated by centrifugation and dissolved in 20% glycerol (Sharkey *et al.*, 1991). With the assay buffer described, the initial lag in the reaction reported by others (Ward and Keys, 1989; Sharkey *et al.*, 1991) was not observed.

#### Chlorophyll and protein analysis

Total Chl, Chla, and Chlb in 80% acetone extracts of frozen triplicate subsamples were determined according to Arnon (1949), who used the extinction coefficients for Chla ( $16.75\ \text{l g}^{-1}\text{ cm}^{-1}$  and  $82.04\ \text{l g}^{-1}\text{ cm}^{-1}$  at 645 nm and 663 nm, respectively) and Chlb ( $45.61\ \text{l g}^{-1}\text{ cm}^{-1}$  and  $9.27\ \text{l g}^{-1}\text{ cm}^{-1}$  at 645 nm and 663 nm,

respectively) given by MacKinney (1941). The soluble proteins were extracted by grinding frozen leaf subsamples to a fine powder in 50 mM *N*-[tri(hydroxymethyl)methyl] glycine (Tricine) buffer (pH 8.0), 2 mM EDTA, 10 mM NaCl, 5 mM MgCl<sub>2</sub>, 75 mM sucrose, 5 mM  $\epsilon$ -aminocaproic acid, 2 mM benzamidine, 8 mM  $\beta$ -mercaptoethanol (+ $\beta$ me), and 2 mM PMSF for 5 min on ice. This was followed by centrifugation at 12 500 *g* at 4 °C for 30 min. Protein concentrations were measured in the decanted supernatant (Bradford, 1976), and 5 vols of cold acetone were added to an aliquot containing 200 mg of protein, which was left overnight in the freezer. The samples were then centrifuged at 12 000 *g* at 4 °C for 15 min. The acetone was allowed to evaporate off. The precipitates were dissolved in 65 mM TRIS-HCl (pH 6.8), 3 M sucrose, 0.6 M  $\beta$ me, 5% sodium dodecylsulphate (SDS, w/v), and 0.01% bromophenol blue at 96 °C for 7 min. The samples were then cooled to room temperature and aliquots of the SDS-dissociated extracts, containing 15  $\mu$ g of protein, were loaded onto a 12.5% SDS–polyacrylamide gel (Mini-Protean 3 Cell, Bio Rad). This protein amount was within the range of linear response of optical density to the concentration of BSA standard (66 kDa), according to previous calibration measurements. The solubilized proteins were separated by SDS–PAGE (Laemmli, 1970) using a 0.75 mm thick gel (12.5% resolving, 4% stacking). Electrophoresis was carried out at room temperature at a constant 200 V. The gels were fixed in 500:150:75 (v/v/v) water–methanol–acetic acid mixture for 75 min, stained in EZ Blue Gel Staining (Sigma) solution for 2 h, and subsequently rinsed in water to remove excess stain. Finally, the gels were scanned with a high-resolution scanner (Scanjet G4050, Hewlett Packard, Spain) and the amount of Rubisco subunits was determined by densitometry with image analysis software (Image Quant, Molecular Dynamics, GE Healthcare, Spain). Alternatively, when electrophoresis with non-reducing (– $\beta$ me) gels was performed, the frozen leaf samples were extracted in buffer without  $\beta$ me containing 10 mM iodoacetamide to prevent the formation of disulphide bonds, before the addition of SDS loading buffer without  $\beta$ me and boiling. To a separate aliquot,  $\beta$ me was added to a final concentration of 0.6 M, as a control of the same samples under reducing (+ $\beta$ me) conditions (Marín-Navarro and Moreno, 2006).

Following electrophoresis, additional gels were blotted for 75 min to PVDF membranes (Bio-Rad, Madrid, Spain) pre-wetted in methanol and equilibrated in 25 mM TRIS, 192 mM glycine,

20% methanol, and 0.1% SDS (pH 8.3) using an electrotransfer cell (Mini Trans-Blot, Bio-Rad, Madrid, Spain) at 400 mA. The blots were blocked immediately following transfer in 2% ECL Advance blocking reagent (GE Healthcare, Barcelona, Spain) in 20 mM TRIS, 137 mM NaCl (pH 7.6) with 0.1% (v/v) Tween-20 (TBS-T) for 1 h at room temperature with shaking. Blots were briefly rinsed twice in TBS-T, then probed with 1:50 000 diluted polyclonal antibody specific for the large Rubisco subunit (Rubisco quantitation kit, Agrisera, Vännäs, Sweden) for 1 h at room temperature with shaking. The antibody solution was decanted and the blot was briefly rinsed twice, and then washed once for 15 min and three times for 5 min in TBS-T at room temperature with shaking. Next, the blots were incubated in secondary antibody (anti-chicken Ig Y peroxidase conjugate, Sigma, Spain) diluted at 1:160 000 in 2% ECL Advance blocking solution for 1 h at room temperature with shaking. The blots were then washed as above and developed for 5 min with ECL Advance detection reagent according to the manufacturer's instructions. Images of the blots were obtained using a CCD imager (Fluor-S Multilimager, Bio-Rad).

#### Statistical analyses

One-way analyses of variance with sampling time as a factor were carried out with the GenStat 6.2 (VSN International Ltd, Hemel Hempstead, UK) statistical software. From these analyses, the standard errors of the differences (SEDs), and the least significant differences of means (three replicates) at  $P < 0.05$  probability were derived. The latter were used for the inspection of differences among values for each sampling time. The homogeneity of variance and the significance of the analysis were not modified appreciably by using arcsine-square root transformation of percentage variables (Rubisco activation and percentage in soluble protein). Therefore, the untransformed data were used.

## Results

### Water and Chl contents

Leaf RWC (Table 1) increased sharply from 3.4% to 87.0% during the first 12 h of rehydration, and additional water

**Table 1.** Relative water contents (RWC), Chl contents, and Chl fluorescence parameters in leaves of *Xerophyta scabrida* during rehydration

Chl fluorescence parameters in the light were measured at 1500  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> light intensity.

Parameter	Time of rehydration (h)						P	SED
	0	12	24	48	72	96		
RWC (%)	3.4 a	87 b	92 c	96 d	97 e	100 f	<0.001	0.04
Chla (mg g <sup>-1</sup> t. wt)	0.010 a	0.014 a	0.047 a	0.19 b	0.29 b	0.43 c	<0.001	0.054
Chlb (mg g <sup>-1</sup> t. wt)	0.039 a	0.049 a	0.043 a	0.070 a,b	0.11b	0.17 c	<0.001	0.023
Chla:Chlb	0.13 a	0.18 a	1.1 b	2.7 c	2.6 c	2.5 c	<0.001	0.24
Chl a+b (mg g <sup>-1</sup> t. wt)	0.049 a	0.063 a	0.090 a	0.26 b	0.41 b	0.60 c	<0.001	0.074
$F_o$		0.015 a	0.13 c	0.090 b	0.095 b	0.091 b	<0.001	0.012
$F_m$		0.020 a	0.25 b	0.36 c	0.42 c	0.45 c	<0.001	0.037
$F_v/F_m$		0.21 a	0.44 b	0.75 c	0.77 c	0.80 c	<0.001	0.085
$F_q'/F_m'$			0 a	0.047 b	0.086 c	0.099 c	0.004	0.017
$F_v'/F_m'$			0.28 a	0.50 b	0.53 b	0.56 b	0.041	0.072
$F_q'/F_v'$			0 a	0.10 b	0.16 b	0.18 b	0.01	0.034
qL			0 a	0.060 a	0.081 a	0.088 a	0.064	0.025
$\Phi_{NPQ}$			0.60 a	0.67 a	0.69 a	0.67 a	0.26	0.034
$\Phi_{NO}$			0.40 b	0.28 a	0.23 a	0.24 a	0.017	0.037

P, probability in the analysis of variance; SED, standard error of the difference among means ( $n=3$ ); within each row, values with the same letter are not significantly different; t. wt, turgid weight.

was gained until full turgor was reached at 96 h after the start of rehydration. Concomitant changes in specific leaf area, with a maximum of  $\sim 0.17 \text{ cm}^2 \text{ mg}^{-1}$  dry weight by 12 h and little further change, have been reported previously (Tuba *et al.*, 1993b). Chl contents (Table 1) were low in desiccated leaves, Chl*b* being relatively more abundant than Chl*a*. Chl contents rose during the rehydration period, with a faster increase after 24 h. Chl*a* accumulated to a greater extent than Chl*b*, such that the Chl*a*:Chl*b* ratios, which were initially  $<1$ , reached a value  $>2.5$ —within the range found in other plants—by 48 h.

### Chl fluorescence

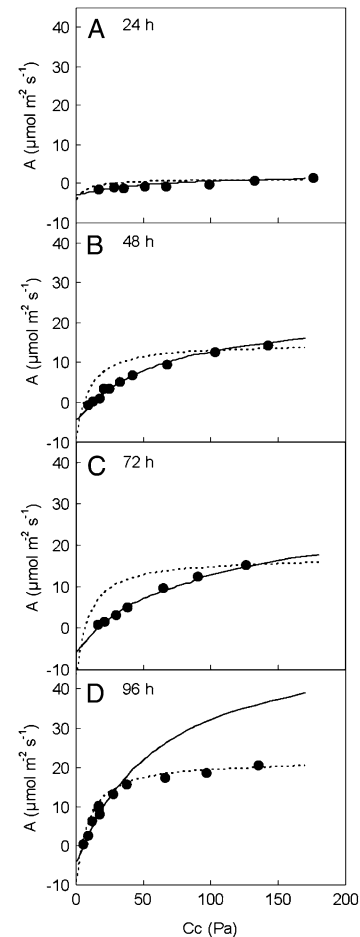
The maximum quantum efficiency of PSII photochemistry ( $F_v/F_m$ , Table 1) increased from 12 h to 48 h of rehydration and changed little thereafter. This change was a consequence of a small increase in  $F_o$  and a large increase in  $F_m$ . The lack of variable fluorescence in the light-adapted state prevented the determination of the fluorescence parameters until 24 h of rehydration. In illuminated leaves ( $1500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ), the PSII operating efficiency and the efficiency factor ( $F_q'/F_m'$  and  $F_q'/F_v'$ , respectively; Table 1) underwent increases from 24 h to 72 h of rehydration, while  $F_v'/F_m'$  rose from 24 h to 48 h with little further change, as was the case for  $F_v/F_m$ . The pattern of change with time in the fraction of open PSII centres (qL) was similar to that in  $F_q'/F_v'$ , but with lower absolute values. The quantum yield of non-photochemical quenching ( $\Phi_{\text{NPQ}}$ , Table 1) underwent little change during the rehydration period, while the quantum yield of non-radiative decay ( $\Phi_{\text{NO}}$ ) decreased by 43% from 24 h to 72 h. By comparison, a rise in NPQ was observed in this interval (data not shown).

### CO<sub>2</sub> fixation

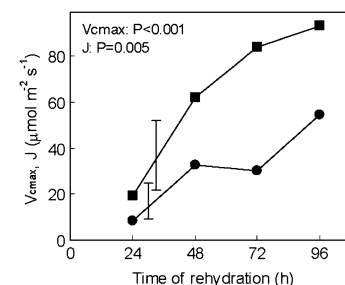
Regardless of the CO<sub>2</sub> concentration used in measurements, there was no CO<sub>2</sub> uptake at 24 h of rehydration (Fig. 1A) or before. Photosynthesis increased in the following 3 d rehydration period and was Rubisco limited up to very high chloroplast CO<sub>2</sub> concentrations at 48 h and 72 h (Fig. 1B, C). The transition from Rubisco-limited to RuBP regeneration-limited photosynthesis decreased to 33 Pa CO<sub>2</sub> partial pressure at 96 h (Fig. 1D), which is still high in comparison with other plants.  $V_{\text{cmax}}$  and  $J$  were calculated (Fig. 2) from the photosynthesis–CO<sub>2</sub> response curves. Except for a drop in  $V_{\text{cmax}}$  at 72 h, which can be attributed to a variation between samples, both  $J$  and  $V_{\text{cmax}}$  increased from 24 h to 96 h, without reaching a plateau. There were relatively higher increases in  $J$  than in  $V_{\text{cmax}}$ .

### Rubisco activity and contents

No Rubisco activity was detected in assays carried out at room temperature, but activity was recorded when the enzyme activation and assays were performed at 35 °C (Fig. 3A). Both the initial and total Rubisco activities increased during the 96 h hydration period, with a faster



**Fig. 1.** Photosynthetic responses of *Xerophyta scabrida* leaves to the CO<sub>2</sub> concentration inside the chloroplast during rehydration. Observed data (filled circles); Rubisco-limited photosynthesis (solid line); RuBP regeneration-limited photosynthesis (dotted line). Measurements were performed under  $1500 \mu\text{mol m}^{-2} \text{ s}^{-1}$  irradiance, at 25 °C and  $1.6 \pm 0.23 \text{ kPa}$  vapour pressure deficit. The statistical significance of the parameters derived from this figure is shown in Fig. 2.



**Fig. 2.** Maximum carboxylation rate allowed by Rubisco,  $V_{\text{cmax}}$  (filled circles), and the rate of photosynthetic electron transport,  $J$  (filled squares), in *Xerophyta scabrida* leaves during rehydration. Vertical bars represent least significant differences between means ( $n=3$ ).

rise—from 12% to 75% final, total Rubisco activity—during the first 12 h. The activation of the enzyme was 44–64% in the first 48 h and increased to 74–83 % in the last 2 d.

Rubisco protein amounts (Fig. 3B) were quantified by SDS-PAGE densitometric analysis of samples extracted with  $+\beta$ me (see Materials and methods). There was little change in the amount of Rubisco protein during the first 48 h of rehydration, although this was followed by an increase of  $\sim 56\%$ . Similarly, total soluble protein remained unchanged for 48 h and then increased by  $\sim 18\%$  (Fig. 3B). As a fraction of soluble protein, Rubisco was relatively low initially and increased (from 19% to 27%) in the last 2 d of rehydration. When Rubisco protein contents and *in vitro* and *in vivo* estimated ( $V_{\text{cmax}}$ ) total Rubisco activities were compared (Fig. 4), it was found that the continued increase in the latter was accompanied by relatively smaller changes in total *in vitro* Rubisco activity after 12 h of rehydration and by an increase in Rubisco protein only after 48 h.

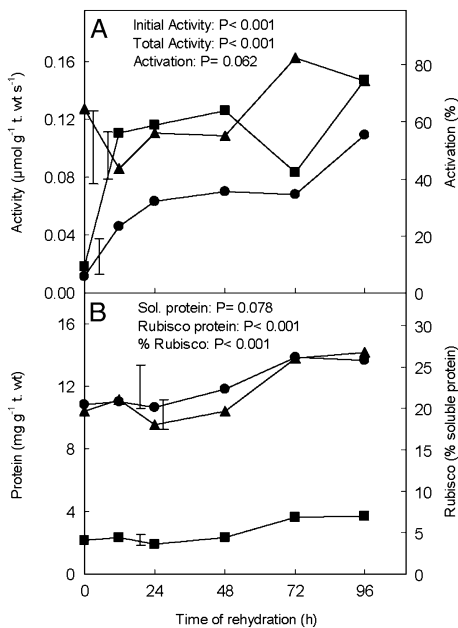
To examine further the disparity between  $V_{\text{cmax}}$  and Rubisco contents, the electrophoresis of samples extracted either with  $+\beta$ me or with iodoacetamide and  $-\beta$ me to block the sulphhydryl groups was compared. Whereas with the reducing agent the two Rubisco subunits migrated according to their molecular weights (Fig. 5), with blocked sulphhydryl groups the Rubisco remained at the top of the gel. In separate aliquots of the same samples to which  $\beta$ me had been added following iodoacetamide treatment, the large Rubisco subunit showed normal migration in the gels, but the small Rubisco subunit was not observed. In comparison, wheat samples extracted with  $\beta$ me or with

iodoacetamide with either  $+\beta$ me or  $-\beta$ me showed few electrophoretic differences, the two Rubisco subunits undergoing normal migration. The examination of the iodoacetamide  $-\beta$ me and  $+\beta$ me gel electrophoresis (Fig. 6) revealed that in both desiccated and rehydrating *X. scabrida* leaves Rubisco formed high molecular weight aggregates. It was therefore investigated whether the reversible aggregation of Rubisco might account for the differences between the gas exchange measurements and Rubisco contents. Leaves harvested at each sampling time were analysed immediately after illumination with bright light in moistened air, rather than after an equilibration period in water after the photosynthesis measurements (see Materials and methods). In these leaves, Rubisco remained in an aggregated state (data not shown).

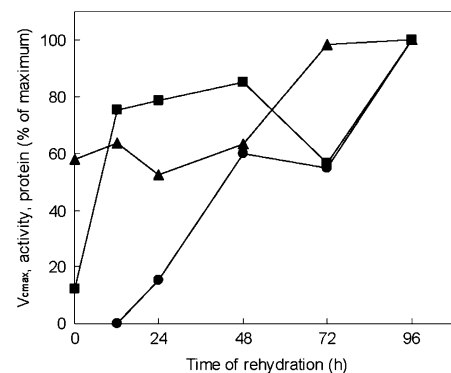
## Discussion

The rehydration of leaves and the recovery of Chl contents in this experiment were in general agreement with earlier reports on poikilochlorophyllous resurrection plants (Tuba *et al.*, 1994; Proctor and Tuba, 2002; Degl'Innocenti *et al.*, 2008). It may be remarked that Chl**b** was relatively more abundant than Chl**a** in desiccated leaves, but by 48 h the Chl**a**:Chl**b** ratio was  $>2.0$ . This suggests a rise during rehydration in the PSII reaction centres with respect to the antenna complexes (Habash *et al.*, 1995; Bailey *et al.*, 2001), which is consistent with the reported up-regulation of PSII genes (Ingle *et al.*, 2007, 2008).

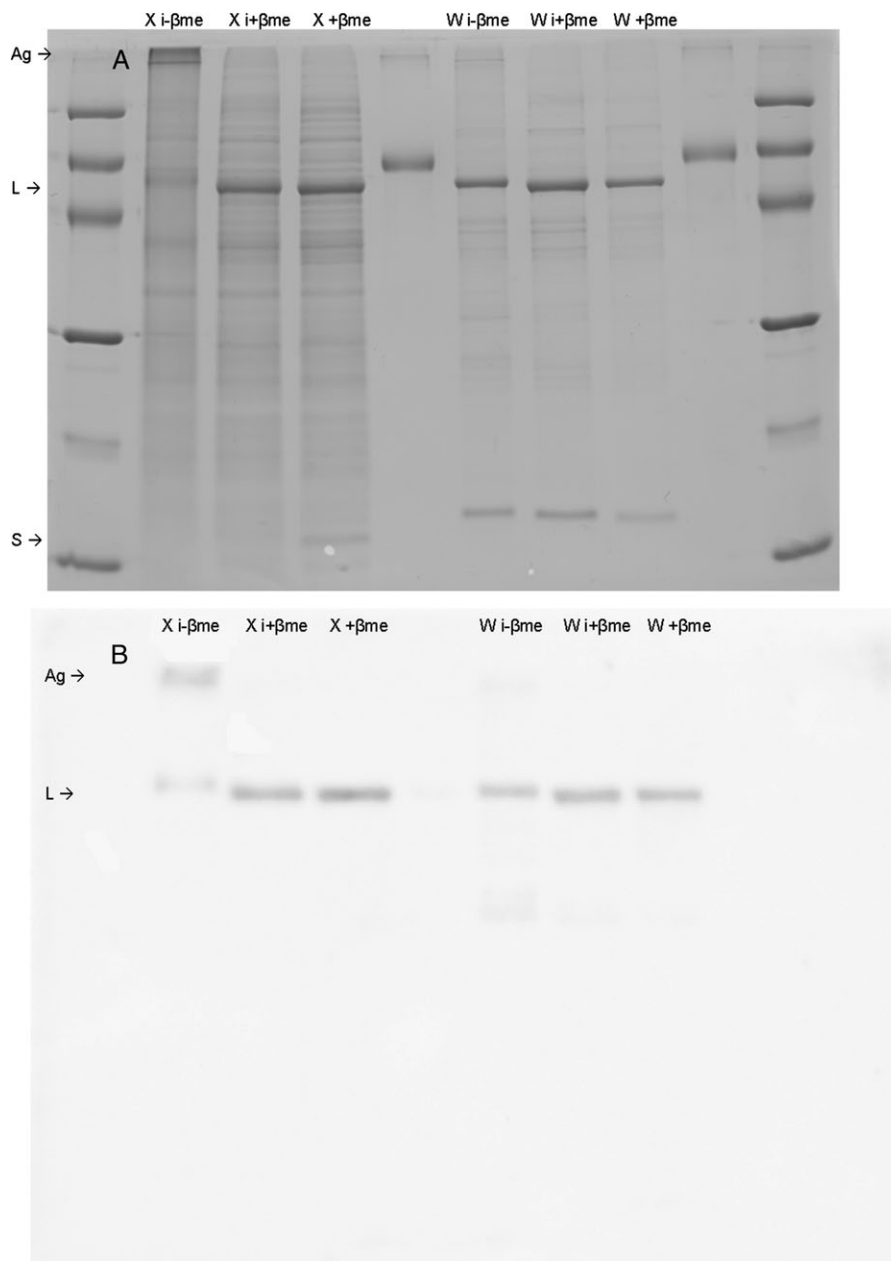
The PSII operating efficiency ( $F_q'/F_m'$ ), in contrast to  $F_v/F_m$  and  $F_v'/F_m'$ , was still relatively low at 48 h after the start of rehydration and continued to increase up to the following day, as did the PSII efficiency factor.  $F_q'/F_v'$  is generally much more affected by the ability to utilize the products of the electron transport chain than by changes in NPQ (Baker *et al.*, 2007). This points to the capacity for carbon assimilation as the factor that limits the efficiency at which the light absorbed by PSII is used for photochemistry in rehydrating *X. scabrida* leaves. A similar conclusion was reached for the HDT *H. rodopensis* during dehydration (Georgieva *et al.*, 2005).  $F_q'/F_v'$  is non-linearly related to



**Fig. 3.** Changes during rehydration of *Xerophyta scabrida* leaves in (A) initial (filled circles) and total (filled squares) Rubisco activities and Rubisco activation state (filled triangles) measured at 35 °C; and (B) Rubisco (filled squares) and total soluble proteins (filled circles) and amount of Rubisco as a percentage of soluble protein (filled triangles). Rubisco was quantified by densitometric analysis of  $+\beta$ me SDS-PAGE. Values are expressed on a turgid weight basis. Vertical bars represent least significant differences between means ( $n=3$ ).



**Fig. 4.** Effect of rehydration of *Xerophyta scabrida* leaves on  $V_{\text{cmax}}$  (filled circles), *in vitro* total Rubisco activity (filled squares), and Rubisco protein (filled triangles), as a percentage of values at 96 h.

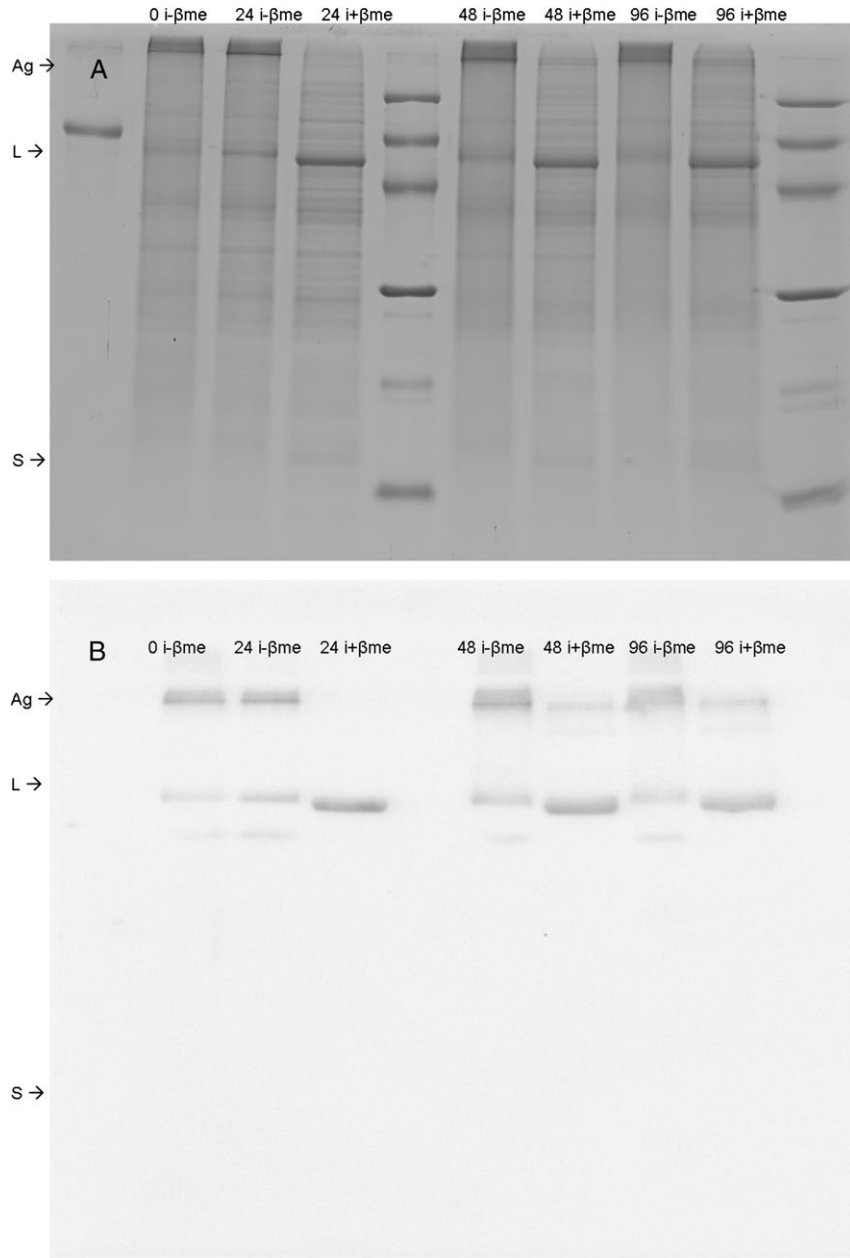


**Fig. 5.** Soluble proteins in desiccated *Xerophyta scabrida* (X) and fresh wheat (W) leaves. (A) SDS-PAGE and (B) immunoblotting of samples extracted in buffer containing 10 mM iodoacetamide without  $\beta$ -mercaptoethanol (i- $\beta$ me), containing iodoacetamide to which  $\beta$ me had been added prior to electrophoresis (i+ $\beta$ me), or with  $\beta$ me without iodoacetamide (+ $\beta$ me). Molecular markers were loaded onto lanes 1 and 10 and BSA standard onto lanes 5 and 9. Rubisco aggregates (Ag) and large (L) and small (S) subunits are indicated on the left.

the proportion of PSII centres that are in the open state (with the primary quinone electron acceptor of PSII,  $Q_A$ , oxidized), as estimated by  $qL$  (Baker *et al.*, 2007). Changes during rehydration in this parameter and  $F_q'/F_v'$  were parallel and showed an increasing fraction of open PSII centres as the carboxylation capacity increased. In contrast to previous reports on the engagement of non-photochemical energy dissipation upon the remoistening of DT leaves (Csintalan *et al.*, 1999; Augusti *et al.*, 2001; Degl'Innocenti *et al.*, 2008), the lack of significant changes in  $\Phi_{NPQ}$  suggests that the down-regulation of PSII was not a major cause of

the changes observed during rehydration in the maximum efficiency of PSII in the light. The significant decline in  $\Phi_{NO}$  probably reflects the reconstitution of functional PSII antennae and reaction centres.

The capacity for carbon assimilation was also recovered during rehydration, although the assimilation rates could differ from those of intact plant leaves due to signalling and metabolic interactions with other organs. In agreement with the results concerning Chl fluorescence, the responses of carbon assimilation to the  $CO_2$  concentration in the chloroplast (Figs 1, 2) indicated that the recovery of



**Fig. 6.** Soluble proteins in *Xerophyta scabrida* leaves after different rehydration periods. (A) SDS-PAGE and (B) immunoblotting of samples extracted in buffer containing 10 mM iodoacetamide without  $\beta$ -mercaptoethanol (i- $\beta$ me), or containing iodoacetamide to which  $\beta$ me had been added prior to electrophoresis (i+ $\beta$ me). Leaves desiccated (0) or rehydrated for 24 h (24), 48 h (48), or 96 h (96). Molecular markers were loaded onto lanes 5 and 10, and BSA standard onto lane 1. Rubisco aggregates (Ag) and large (L) and small (S) subunits are indicated on the left.

photosynthetic capacity during rehydration was relatively more limited by carboxylation than by the rate of electron transport. Rubisco activity may therefore be of paramount importance for the photosynthetic competence of rehydrated desiccation-tolerant plants. The present results show that a significant fraction of the Rubisco protein found in rehydrated leaves is present in desiccated leaves (Fig. 3), and that new synthesis occurs later in the process of rehydration. Notably, in both desiccated and rehydrated *X. scabrida* leaves—in contrast to wheat—Rubisco was in an aggregated state (Fig. 5), as in *L. minor* fronds under

osmotic stress (Ferreira and Shaw, 1989). Our gas exchange measurements and Rubisco activity assays revealed that free or membrane-bound Rubisco aggregates in *X. scabrida* were inactive in desiccated leaves and in the early rehydration stages. Treatments such as high light intensity in gas exchange analysis or mild warming under reducing conditions in activity assays rendered Rubisco progressively more active. This suggests that the integrity of Rubisco was preserved in the aggregates, but that a modification required for the enzyme to become functional was facilitated by rehydration. In dormant *Retama raetam* tissues, Rubisco



and other proteins also appear to be present as high molecular weight complexes (Pnueli *et al.*, 2002). These complexes precipitated during extraction with reducing buffers, a result that was observed for the small Rubisco subunit only when  $\beta$ me was added to extracts containing iodoacetamide. Pnueli *et al.* (2002) suggested that the dilution of reducing equivalents upon rehydration releases proteins from the aggregates into their soluble, active form. However, some of the DTT concentrations used by Pnueli *et al.* (2002) in the protein extraction buffer have been shown to cause Rubisco aggregation and precipitation (Cho *et al.*, 2008). Moreover, the present results suggested that the increase in Rubisco activity during rehydration was not associated with protein release from the aggregates. The lower oxidation states of thiol groups (disulphide and sulphenic acid) may easily be reverted again to the sulphhydryl state by disulphide exchange with free thiols, by DTT (*in vitro*) or by thioredoxins and glutaredoxins (Marcus *et al.*, 2003; Moreno *et al.*, 2008). It is possible that oxidative conditions during desiccation could induce the formation of disulphides in the Rubisco molecule, and that the recovery of photochemical activity could lead to an increasingly reduced stroma, favouring the reductive activation of Rubisco. While upon desiccation of *X. scabrada*, and indeed of all poikilochlorophyllous plant species, Chl and the photosynthetic apparatus are lost, it is concluded that Rubisco is preserved in large amounts in a close to functional state. Rubisco aggregation may be a part of the poikilochlorophyllous strategy.

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