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ORIGINAL ARTICLE

Metabolic processes sustaining the reviviscence of lichen *Xanthoria elegans* (Link) in high mountain environments

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Abstract To survive in high mountain environments lichens must adapt themselves to alternating periods of desiccation and hydration. Respiration and photosynthesis of the foliaceous lichen, *Xanthoria elegans*, in the dehydrated state were below the threshold of CO_2-detection by infrared gas analysis. Following hydration, respiration totally recovered within seconds and photosynthesis within minutes. In order to identify metabolic processes that may contribute to the quick and efficient reactivation of lichen physiological processes, we analysed the metabolite profile of lichen thalli step by step during hydration/dehydration cycles, using ^{31}P- and ^{13}C-NMR. It appeared that the recovery of respiration was prepared during dehydration by the accumulation of a reserve of gluconate 6-P (glcn-6-P) and by the preservation of nucleotide pools, whereas glycolytic and photosynthetic intermediates like glucose 6-P and ribulose 1,5-diphosphate were absent. The large pools of polyols present in both *X. elegans* photo- and mycobiont are likely to contribute to the protection of cell constituents like nucleotides, proteins, and membrane lipids, and to preserve the integrity of intracellular structures during desiccation. Our data indicate that glcn-6-P accumulated due to activation of the oxidative pentose phosphate pathway, in response to a need for reducing power (NADPH) during the dehydration-triggered down-regulation of cell metabolism. On the contrary, glcn-6-P was metabolised immediately after hydration, supplying respiration with substrates during the replenishment of pools of glycolytic and photosynthetic intermediates. Finally, the high net photosynthetic activity of wet *X. elegans* thalli at low temperature may help this alpine lichen to take advantage of brief hydration opportunities such as ice melting, thus favouring its growth in harsh high mountain climates.

Keywords Energy metabolism · Lichens · Metabolic profiling · NMR spectroscopy · Reviviscence · *Xanthoria elegans*

Abbreviation

CDTA	1,2-Cyclohexylenedinitrilotetraacetic acid
Glcn-6-P	Gluconate 6-P
GPG	Glycerophosphoglycerol
GPC	Glycerophosphocholine
PCA	Perchloric acid

Introduction

Lichens are among the most resistant of living organisms. They survive in extreme environments including the deserts and frigid areas of all five continents. In the alpine environment, these symbiotic organisms are exposed to harsh fluctuations in water supply, light intensity, and temperature (Kappen 1988; Körner 2003). *Xanthoria elegans* (Link), used in this study, is a lichen typical of this habitat, whose growth on rocky surfaces mainly depends on atmospheric water inputs. *X. elegans* is a saxicolous desiccation-tolerant lichen of the *Teloschistaceae* family (Helms 2003),

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containing an ascomycetous fungus and a unicellular green alga belonging to the *Teloschistes* and *Trebouxia* genera, respectively (Helms 2003). At the site where they were harvested thalli naturally undergo hydration/dehydration cycles, growing when they are hydrated by melting snow, rain, or dew.

Metabolic activity of lichen thalli, apparent as gas exchange, becomes almost undetectable when their water content decreases below 10–15% of their dry weight (Lange 1980; Schroeter et al. 1991). But lichens may take up sufficient moisture from atmospheric water vapour (Lange 1980) to stay above this low hydration threshold, and as such maintain significant metabolic activity even under quite dry conditions. Hydration is facilitated by high concentrations of polyols in both the photo- and mycobiont (Rundel 1988) that lower water potential (Lange et al. 1990) allowing photosynthetic activity even under increasing degrees of desiccation (Nash et al. 1990). Immersion in water or rewetting in moist air triggers the process of reviviscence in dry desiccation-tolerant lichens (Smith and Molesworth 1973; Bewley 1979). Respiration and photosynthesis quickly return to normal upon hydration, indicating that cell damage induced by drying is rapidly repaired (Farrar and Smith 1976). According to several authors (Dudley and Lechowicz 1987; Longton 1988), desiccated membranes are leaky, leading to the loss of organic and inorganic solutes from the cell into the surrounding solution during rewetting. Thus, cell survival requires a rapid resealing of plasma-membrane disruptions (McNeil and Steinhardt 1997; McNeil et al. 2003). Nevertheless, some delay before repair and synthesis of lost metabolites should be expected.

Intense solar radiation is another environmental stress that threatens lichens with the potential damaging effects of reactive oxygen species (ROS) production (Weissman et al. 2005), in particular when photosynthetic water oxidation stops due to dehydration. In the plant kingdom, the ability to withstand light stress and desiccation of vegetative organs involves various mechanisms recently reviewed by Hoekstra et al. (2001), Rascio and La Rocca (2005), and Heber et al. (2005). In hydrated organs, these include increased energy dissipation by fluorescence at PSII, and cyclic electron transport around PSI (Heber and Walker 1992; Manuel et al. 1999; Cornic et al. 2000). On the contrary, when they are dry, poikilohydric organisms do not emit light-induced fluorescence, revealing inactivation of PSII function (Lange et al. 1989). These organisms minimise damage by activating transfer to non-photochemical quenchers and converting excess energy to heat (Bilger et al. 1989; Heber et al. 2000). Interestingly, following hydration, a burst of intracellular production of ROS occurs in the photo- and mycobiont of *Ramalina lacera*, which modifies superoxyde dismutase, catalase, glutathione reductase, and glucose 6-P dehydrogenase activities (Weissman et al. 2005).

In desiccation-tolerant plants, the repair of cell damage upon hydration, and the subsequent restoration of cell function, involves various complex inductive mechanisms including gene transcriptions and an increased protein turnover (Oliver et al. 2004), therefore it requires a delay ranging from a few hours to several days (Gaff 1997). In contrast, we observed that respiration and photosynthetic activity of several high-mountain lichens restarted almost immediately after rewetting. Therefore, we hypothesised that the rapid recovery of these organisms relies on the preservation/accumulation of key pools of metabolites during dehydration. We supposed, for example, that metabolic intermediates like respiratory substrates, nucleosides, and pyridine nucleotides, do not significantly leak from the photo- and mycobiont during hydration/desiccation cycles in lichens. To substantiate this hypothesis, we assessed in parallel the respiration and photosynthetic activities and the metabolite profiles of *X. elegans* thalli during hydration and dehydration. The main pools of metabolites were characterised in vitro and in vivo using ^{31}P- and ^{13}C-NMR, as a convenient technique giving a precise overview of the soluble organic compounds present in plant materials (Bligny and Douce 2001; Streb et al. 2003).

Materials and methods

Lichen sampling and measurement of dry and wet weight

Xanthoria elegans thalli were collected at 2,800 m on limestone rocks situated above the Galibier pass (Hautes-Alpes), an area characterised by a relatively continental and dry climate. The so-called "dry thalli" were taken under the sun, during the hottest hours of the day (rocks surface temperature 30–35°C; relative air humidity 20%). They still contained ca 8% water by comparison with oven-desiccated thalli (2 h at 110°C). The so-called "wet thalli" correspond to lichens collected in the same area either at dawn, in the dark, or just after a rain, in the light. The weight of wet thalli was measured in situ after removing interstitial water by straining between two layers of absorbent paper. The wet weight versus dry weight ratio was usually ca 2.5. Thalli were stored frozen at −20°C until needed.

Measurements of respiration and photosynthesis activities

These activities were measured either through O_2 or CO_2 exchange. CO_2 exchange was measured with an infrared gas analyser (IRGA, LI-COR 6200, Lincoln, USA) equipped with a 1.2 l chamber. The temperature inside the closed chamber was thermostatically maintained between ~2.5°C and 30°C. Thalli (ca 0.5 g dry weight) were left attached to their support. O_2 was monitored polarographically

at 20°C in a 1 ml water cuvet equipped with a Clark-type oxygen-electrode purchased from Hansatech Ltd (King's Lynn, Norfolk, UK). The O_2 concentration in an air- saturated medium was taken as 210 µM at 20°C and 780 hPa in the alpine lab (2,100 m) where measurements were done. To enable the sample to be stirred in the measurement chamber, thalli were fragmented into 1–2 mm^2 pieces by gentle grinding with mortar and pestle in liquid nitrogen; 50 mg of dry thalli was used for O_2-electrode measurements. Controls were made to ensure that lichen fragmentation in liquid nitrogen did not modify the rates of respiration and photosynthesis after unfreezing and hydration. I A saturating radiation dose of photosynthetically active photon flux density (PPFD) of 500 µmol m^{-2} s^{-1}, was provided using a Shott KL 1500 (Amilabo, Chassieu, France) light generator. The incubation medium contained 0.1 mM potassium bicarbonate, pH 6.8.

Perchloric Acid (PCA) extract preparation

Thalli (4 g dry weight) were quickly frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle with 1 ml of 70% (vol/vol) PCA. The frozen powder was kept at −10°C until use. The thick suspension thus obtained was centrifuged at 15,000*g* for 10 min to remove particulate matter and the supernatant was buffered with 2 M $KHCO_3$ to about pH 5.2. The supernatant was then centrifuged at 10,000*g* for 10 min to remove $KClO_4$; the resulting supernatant was lyophilised and stored in liquid nitrogen. This freeze-dried material was dissolved in 2.5 ml of water containing 10% D_2O, and stored frozen.

^{31}P- and ^{13}C-NMR analyses of PCA extracts

Spectra were recorded on a Bruker NMR spectrometer (AMX 400, wide bore; Bruker Instruments, Inc., Billerica, MA, USA) equipped with a 10 mm multinuclear probe tuned at 162 or 100.6 MHz for ^{31}P- or ^{13}C-NMR studies, respectively. The deuterium resonance of D_2O was used as a lock signal.

^{13}C-NMR acquisition conditions: 90° radio frequency pulses (19 µs) at 6 s intervals; spectral width 20,000 Hz; 3,600 scans; Waltz-16 ^{1}H decoupling sequence (with two levels of decoupling: 2.5 W during acquisition time, 0.5 W during delay). Free induction decays were collected as 16-K data points, zero-filled to 32K, and processed with a 0.2 Hz exponential line broadening. ^{13}C-NMR spectra are referenced to hexamethyldisiloxane at 2.7 ppm. Mn^{2+} was chelated by the addition of 2 µmol 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA) and the pH was adjusted to 7.5 (sample volume of 2.5 ml).

^{31}P-NMR acquisition conditions: 70° radio frequency pulses (15 µs) at 3.6-s intervals; spectral width 8,200 Hz; 4,096 scans; Waltz-16 ^{1}H decoupling sequence (with two levels of decoupling: 1 W during acquisition time, 0.5 W during delay). Free induction decays were collected as 8-K data points, zero-filled to 16K and processed with a 0.2-Hz exponential line broadening. ^{31}P-NMR spectra are referenced to methylenediphosphonic acid (pH 8.9) at 16.38 ppm. Before ^{31}P-NMR analyses, divalent cations were chelated by the addition of appropriate amounts of CDTA ranging from 100 to 150 µmol (sample volume of 2.5 ml). The much higher amount of CDTA as compared to ^{13}C-NMR analysis (see above) corresponded to the necessity to chelate all divalent cations including Ca^{2+}, Mg^{2+}, etc. while only Mn^{2+} had to be chelated for ^{13}C-NMR analysis. The pH was buffered by the addition of 75 µmol Hepes and adjusted to 7.5.

The assignments were made after running a series of standard solutions of known compounds at pH 7.5 and adding aliquots of these compounds to the PCA extracts as described previously (Roby et al. 1987). Identified compounds were quantified by comparison of the surface of their resonance peaks to the surface of resonance peaks of standards added to samples before grinding according to Aubert et al. (1996); $NADP^+$ and NADPH were quantified as described by Pugin et al. (1997). Fully relaxed conditions during spectra acquisition (pulses at 20-s intervals) were used for quantification. The standards utilised were methylphosphonate and maleate for ^{31}P- and ^{13}C-NMR analyses, respectively.

In vivo ^{31}P-NMR measurements

A perfusion system was utilised to optimise the signal-to-noise ratio as described earlier (Gout et al. 2001). Spectra were recorded on a Bruker spectrometer (AMX 400, wide bore) equipped with a 25-mm probe tuned at 162 MHz. ^{31}P-NMR acquisition conditions: 50° radio frequency pulses (70 µs) at 0.6 s intervals; spectral width 9,800 Hz; 6,000 scans; Waltz-16 ^{1}H decoupling sequence (with two levels of decoupling: 2.5 W during acquisition time, 0.5 W during delay). Free induction decays were collected as 4-K data points, zero-filled to 8K and processed with a 2 Hz exponential line broadening. Spectra were referenced to a solution of 50 mM methylenediphosphonic acid (pH 8.9 in 30 mM Tris) contained in a 0.8-mm capillary itself inserted inside the inlet tube along the symmetry axis of the cell sample (Roby et al. 1987). The assignment of inorganic phosphate (Pi), phosphate esters, phosphate diesters, and nucleotides to specific peaks was carried out according to Roberts and Jardetzky (1981), Roby et al. (1987), Aubert et al. (1996), and from spectra of the PCA extracts that contained the soluble low molecular weight constituents.

Results

As frequently observed with lichens (Smith and Molesworth 1973; Farrar and Smith 1976; Larson 1981), *X. elegans* can lose most of its constitutive water in a dry atmosphere without apparent damage. The dehydration of this lichen took about 30 min when it was exposed to the summer sun, but it was rehydrated immediately on application of water. Whilst respiration was undetectable in the dry thalli, upon rewetting its initiation and progress to full speed within the seconds was spectacular.

In situ measurement of gas exchanges in *X. elegans* thalli

No gas exchange was detected using the IRGA apparatus (detection threshold, ca 10 nmol CO_2 min^{-1}) in thalli containing less than 10% water, irrespective of the temperature and the light conditions i.e. dark or light (photon flux exceeding 1,500 µmol m^{-2} s^{-1}). This indicated that photosynthesis and respiration activities were negligible. On the contrary, CO_2 uptake or loss, depending on light or dark conditions, was easily measured across a range of temperatures in wet thalli (Fig. 1). Interestingly, chlorophyll fluorescence measured according to Heber et al. (2000) was largely quenched in dry lichens, whereas it was detected under light within the first min following hydration, increasing fast and reaching a steady state after 5–6 min (data not shown). This confirms that electron transport was negligible in dry thalli.

Not surprisingly, CO_2 exchange of wet thalli was temperature-dependent. In the dark, the respiration of wet thalli increased exponentially with temperature (Fig. 1), like that of higher plants (Bligny et al. 1985). For example, at 5°C, the emission of CO_2 was 25 ± 5 nmol O_2 min^{-1} g^{-1} lichen dry weight, whereas it was 600 ± 90 at 20°C. Under the light, the uptake of CO_2 increased with temperature up to a maximum of 800 ± 80 nmol O_2 min^{-1} g^{-1} lichen dry weight at 10–15°C, and then decreased continuously above 15°C. Net CO_2 uptake, considered as net photosynthesis, can be broken-down as gross CO_2 uptake due to the photosynthetic activity of the alga, and the emission of CO_2 mainly due to fungal respiration if we consider that algal respiration was negligible in the light (Gans and Rébeillé 1988; Tcherkez et al. 2005). In illuminated wet thalli, the CO_2 emitted by respiration compensated for the CO_2 assimilated by photosynthesis at 28–29°C.

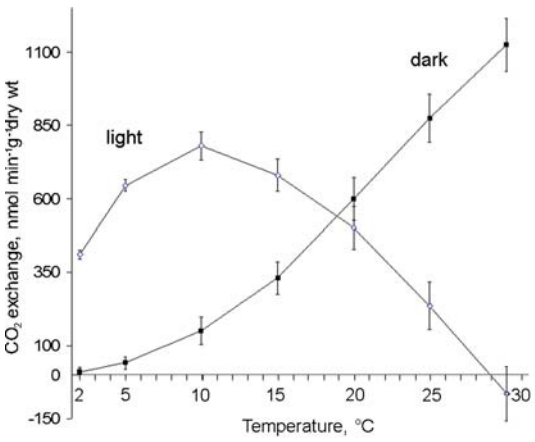

Fig. 1 Respiration (*filled circle*) and net photosynthesis (*open circle*) of wet *X. elegans* thalli in the dark and light at different temperatures. Respiration and net photosynthesis were determined in situ via CO_2 exchanges measured by IRGA, as described in Materials and methods. Values are calculated from the weight of thalli dried at 110°C. Values are means ± SD (n = 5)

Recovery of respiration and photosynthesis activities of dry *X. elegans* thalli after hydration

In the following experiments, gas exchange was measured on immersed thalli fragments with a Clark-type O_2-electrode, at 20°C, as indicated in Materials and methods. We preferred this technique because it was very difficult to stabilise the temperature and relative humidity in the IRGA measurement chamber during dark/light cycles. The oxygraphic traces (Fig. 2) show that: (1) the O_2 uptake by thalli fragments introduced into the dark O_2-electrode chamber started immediately at full speed irrespective of their initial dry or wet status (traces a1 and b1). Oxygen was consumed at a rate of between 580 and 620 nmol min^{-1} g^{-1} lichen dry weight. These values were comparable with the rates of CO_2 production measured in situ with the IRGA. (2) In the light, there was a 1-min delay before the rate of O_2 production initially by wet thalli stabilised (Fig. 2, trace b2). This delay was shorter when the duration of the preceding dark period was reduced (trace b2) and longer (2–3 min) when thalli were initially dry (trace a2). In all cases, the stable rate of O_2 emission was comparable (between 520 and 640 nmol min^{-1} g^{-1} lichen dry weight); (3) when samples were then alternately illuminated and darkened in successive dark/light sequences, respiration and net photosynthesis rates remained remarkably stable over several hours; (4) assays done with lyophilised thalli containing less than 4% water or with thalli kept dry in the laboratory over 1 year gave similar results (not shown), thus showing the remarkable adaptation of this lichen to severe and prolonged desiccation.

Taken together, these data indicate that the cell structures of the fungal and algal partners did not suffer irreversible desiccation-induced damage. Moreover, the fact that respiration recovered instantly suggested that key metabolic substrates, nucleotides, and pyridine nucleotides, were not lost during the hydration/desiccation cycle. On the contrary, the delayed recovery of photosynthesis suggested that key Benson–Basham–Calcin cycle intermediates

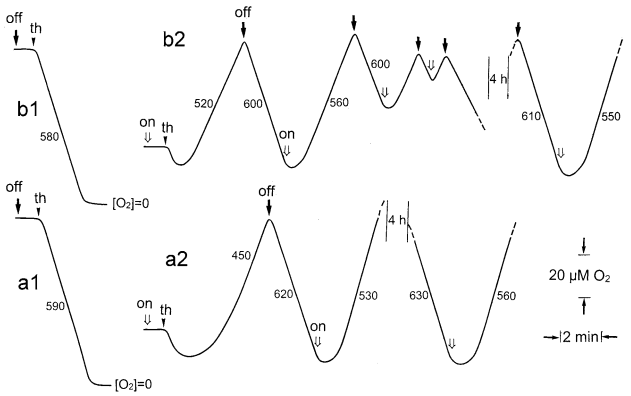

Fig. 2 O_2 exchange in *X. elegans* thalli successively incubated in the dark and saturating light. These traces were obtained using thalli fragments incubated in an oxygen-electrode chamber. Values on the traces refer to nmol of O_2 consumed or produced min^{-1} g^{-1} lichen dry weight. Thalli (*th*) were introduced into the chamber as dry fragments in dark (*a1*) or light (*a2*), or as wet fragments in dark (*b1*) or light (*b2*). The incubation medium contained 0.1 mM bicarbonate at pH 6.8. *Open arrows* light on; *solid arrows* light off

decreased sizably in the chloroplasts of photobiont during thalli dehydration. In order to verify these hypotheses, we analysed the metabolite profiles of dry and wet *X. elegans* thalli, and thalli during rewetting and drying cycles.

Metabolite profiling of *X. elegans* thalli

Perchloric acid extracts of *X. elegans* thalli were analysed using ^{31}P- and ^{13}C-NMR spectroscopy as described in the Materials and methods. Representative spectra are shown in Fig. 3 and 5, and comparative data are given in Table 1.

In dry thalli, the two most abundant metabolites measured by ^{31}P-NMR (Fig. 3a) were inorganic phosphate (1.7 µmol g^{-1} dry weight) and glcn-6-P (0.91 µmol g^{-1} dry weight). Other P-compounds identified were, from downfield to upfield: mannitol 1-P, glycerate 3-P, AMP, $NADP^+$, P-choline, the two phosphodiesters glycerylphosphoryl–glycerol and -choline (GPG and GPC), nucleosides (mainly ATP and ADP), and nucleoside diphosphate sugars, UDP-glc and UDP-*N*-acetylglucosamine (UDP-glcNAc). Polyphosphates were also detected in PCA extracts; however, they often precipitate during PCA extraction and we quantified these compounds from in vivo NMR analyses. Spectra like the one shown on Fig. 4 indicate that thalli contained a 6.5-µmol g^{-1} dry weight Pi equivalent of polyphosphates, which constitutes by far, the largest phosphate pool in this lichen. In vivo assays also indicated that the distribution of Pi between alkaline (pH 7.5 cytoplasm) and acidic (pH 5.0–5.5 vacuoles) compartments was roughly 1:2.

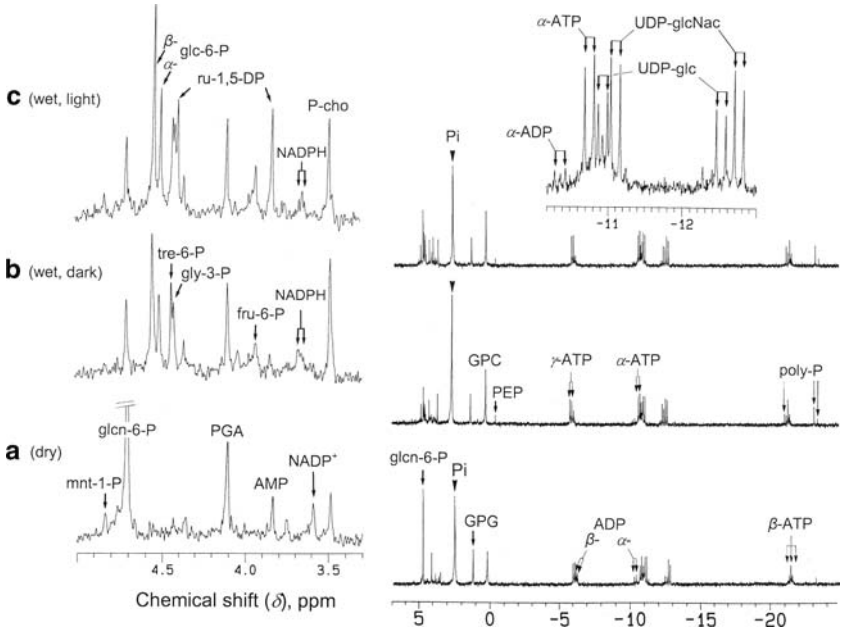

Fig. 3 Proton-decoupled ^{31}P-NMR spectra (161.93 MHz) of perchloric acid extracts of *X. elegans* thalli. Extracts were prepared from 4 g of thalli (on a dry weight basis) and analysed by ^{31}P-NMR. Thalli were collected as follows: **a** dry, in the light; **b** wet, in the dark; **c** wet, in the light. Peak assignments (from downfield to upfield): *mnt-1-P* mannitol 1-P; *glcn-6-P* gluconate 6-P; *glc-6-P* glucose 6-P; *tre-6-P* trehalose 6-P; *gly-3-P* glycerol 3-P; *PGA* phosphoglycerate; *fru-6-P* fructose 6-P; *ru-1,5-DP* ribulose 1,5-diphosphate; *AMP* adenosine monophosphate; *P-cho* P-choline; *GPG* glycerophosphoglycerol; *GPC* glycerophosphocholine; *PEP* phosphoenolpyruvate; *UDP-glc* uridine 5′-diphosphate-α-D-glucose; *UDP-glcNAc* uridine 5′-diphospho-*N*-acetylglucosamine; *poly-P* polyphosphates. Spectra are representative of five independent experiments

Table 1 Metabolic profiles of dry and wet *X. elegans* thalli

Metabolite	Dry lichens (light)	Wet lichens (dark)	*P* value (*t* test)	Wet lichen (light)	*P* value (*t* test)
Sucrose	27 ± 3	29 ± 3	7.01E-01	27 ± 3	3.49E-01
Trehalose	9 ± 2	5 ± 2	1.81E-02*	8 ± 2	2.70E-01
Ribitol	110 ± 10	90 ± 9	3.37E-02*	105 ± 10	5.81E-01
Arabitol	360 ± 30	240 ± 20	9.69E-06*	340 ± 30	3.01E-01
Mannitol	240 ± 20	290 ± 30	2.36E-01	250 ± 20	3.13E-01
Glutamate	45 ± 5	40 ± 4	5.17E-01	43 ± 5	6.19E-01
Glutamine	120 ± 12	71 ± 7	2.43E-05*	125 ± 12	6.50E-02
Choline	85 ± 9	80 ± 8	9.34E-02	88 ± 9	7.15E-01
Betaine	170 ± 15	150 ± 14	2.32E-01	160 ± 15	2.23E-01
Polyphosphates	6.5 ± 1	6.4 ± 1	3.81E-01	6.2 ± 1	4.37E-01
Pi	2.5 ± 0.4	2.7 ± 0.4	3.93E-01	3.6 ± 0.5	1.63E-03*
Mannitol 1-P	0.06 ± 0.01	0.02 ± 0.01	1.62E-03*	0.5 ± 0.01	1.14E-08*
Glcn-6-P	0.91 ± 0.09	0. 18 ± 0.02	4.38E-06*	0.20 ± 0.02	1.79E-06*
Glucose 6-P	nd	0.52 ± 0.05	nd	0.81 ± 0.08	1.43E-04*
Trehalose 6-P	nd	0.22 ± 0.02	nd	0.22 ± 0.02	3.71E-03*
Glycerol 3-P	nd	0.15 ± 0.02	nd	0.17 ± 0.02	1.05E-03*
Ru-1,5-DP	nd	nd	nd	0.27 ± 0.03	nd
PGA	0.27 ± 0.03	0.25 ± 0.03	7.06E-01	0.27 ± 0.03	5.49E-01
Fructose 6-P	nd	0.8 ± 0.01	nd	0.13 ± 0.02	7.15E-04*
AMP	0.12 ± 0.02	0.04 ± 0.01	nd	nd	nd
NADPH	0.02 ± 0.01	0.08 ± 0.01	1.19E-04*	0.08 ± 0.01	2.49E-05*
$NADP^+$	0.05 ± 0.01	nd	nd	nd	nd
P-choline	0.15 ± 0.02	0.40 ± 0.04	1.26E-04*	0.33 ± 0.03	1.78E-04*
GPG	0.30 ± 0.03	0.25 ± 0.02	5.52E-03*	0.25 ± 0.02	1.63E-02*
GPC	0.28 ± 0.03	0.47 ± 0.05	3.55E-03*	0.47 ± 0.05	4.94E-08*
PEP	nd	0.07 ± 0.01	nd	0.06 ± 0.01	3.45E-04*
ATP	0.38 ± 0.04	0.48 ± 0.05	1.40E-01	0.53 ± 0.05	3.81E-02*
ADP	0.14 ± 0.02	0.08 ± 0.01	3.04E-03*	0.05 ± 0.01	1.96E-05*
UDP-glc	0.13 ± 0.02	0.33 ± 0.04	1.68E-05*	0.33 ± 0.04	3.15E-07*
UDP-glcNAc	0.39 ± 0.04	0.43 ± 0.04	1.04E-01	0.46 ± 0.05	4.58E-03*

Dry thalli were collected in the light and wet thalli either in the dark (at dawn) or in the light. Metabolites were identified and quantified from PCA extracts, using maleate and methylphosphonate as internal standards for 13C- and 31P-NMR analyses, respectively, as described in Materials and methods. Values are given as µmol g-1 lichen dry weight. Abbreviations as indicated in the legends of Fig. 3 and 5; nd, not detected (< 0.05 µmol). Values were obtained from a series of independent experiments and are given as mean ± SD ($n = 5$). Statistical *t* test was applied to the data and *P* values are indicated (*$P < 0.05$) for wet lichens (dark) versus dry lichens (light) and for wet lichens (light) versus dry lichens (light)

The metabolite profile of wet thalli harvested in the dark (at dawn) is strikingly different from that of dry ones (Fig. 3b). First, wet thalli contained large pools of various sugar phosphates, including glucose 6-P, trehalose 6-P, and fructose 6-P. Glyceraldehyde 3-P, also phosphoenolpyruvate. Second, glcn-6-P was much less abundant (0.18 µmol g^{-1} dry weight) in wet than in dry thalli. Third, wet thalli contained NADPH ($NADP^+$ was not detected), P-choline, GPC, and ATP (but less ADP and AMP), and their UDP-glc pool was ca three times larger. PGA, GPG, and UDP-glcNAc pools were similar in dry and wet thalli. The metabolite profiles of wet thalli harvested in the light (Fig. 3c) resemble those of wet thalli harvested in the dark, except for a striking double peak, corresponding to the Benson–Basham–Calcin cycle intermediate ribulose 1,5-diphosphate (ru-1,5-DP), located in the chloroplasts of the algal partner. The fluctuations of Pi between samples may originate from partial hydrolyses of polyphosphates during PCA extraction.

^{13}C-NMR spectra show much fewer differences due to the water status of thalli. Typically, dry-thalli spectra (Fig. 5a) contain major resonance peaks corresponding to polyols; namely arabitol, mannitol, and ribitol (360, 240, and 110 µmol g^{-1} dry weight, respectively). The other compounds detected included two sugars, sucrose and trehalose, two amino acids, glutamine and glutamate, choline

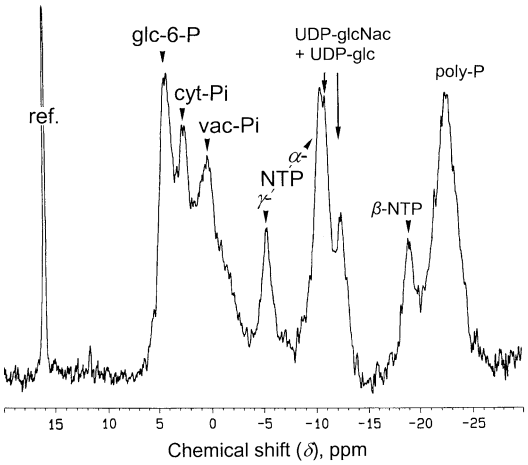

Fig. 4 Proton-decoupled in vivo ^{31}P- NMR spectrum of *X. elegans* thalli. Lichen fragments (4 g dry weight) were hydrated, packed in a 25 mm NMR tube as described in Gout et al. (2001), continuously perfused at a flow rate of 50 ml min^{-1} with a well-oxygenated medium containing 0.2 mM Mops buffer (pH 6.2), at 20°C, and analysed by ^{31}P-NMR. Peak assignments as in Fig. 2; *cyt-Pi* cytoplasmic phosphate; *vac-Pi* vacuolar phosphate; *ref* reference

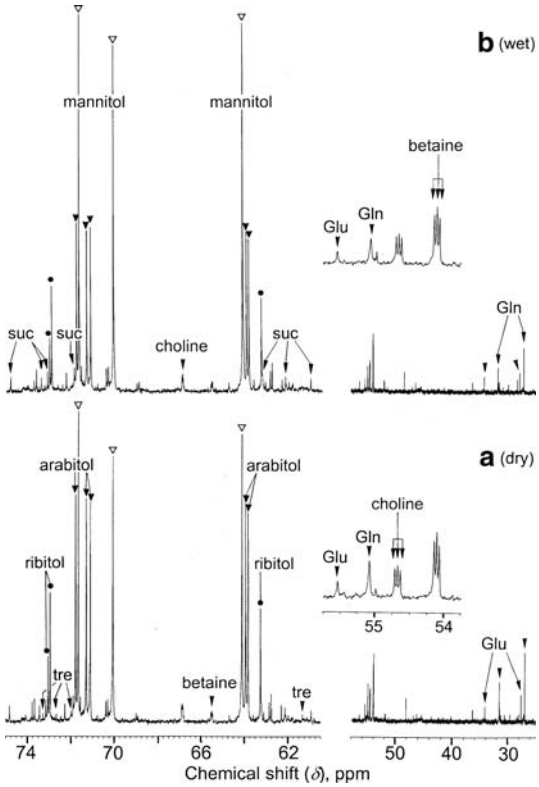

Fig. 5 Proton-decoupled ^{13}C-NMR spectra (100.6 MHz) of perchloric acid extracts of *X. elegans* thalli. PCA extracts were prepared from 4 g of thalli (on a dry weight basis) and analysed by ^{13}C-NMR. Thalli were collected as follows: **a** dry, in the light; **b** wet, in the dark. Peak assignments (from downfield to upfield): *suc* sucrose; *tre* trehalose; *Glu* glutamate; *Gln* glutamine. Spectra are representative of five independent experiments

and its oxidation product betaine. The spectra of wet thalli collected under the light did not significantly differ from those of dry thalli (not shown). In contrast, wet thalli collected at dawn (dark) contained significantly less arabitol and ribitol (240 and 90 µmol g^{-1} dry weight) (Table 1; Fig. 5b), and similar amounts of sucrose, choline, and betaine. Interestingly, arabitol was absent from wet thalli after several days of storing at 20°C in the dark, but mannitol and ribitol remained present (not shown). Wet thalli collected at dawn also contained less glutamine, suggesting that a portion of this amino acid was utilised during the night to sustain the synthesis of nitrogen-containing compounds in wet tissues.

Time course changes in the metabolite profile of *X. elegans* thalli during cycles of hydration and desiccation

Dry thalli fragments were immersed into deionised water at 20°C, in the light, and withdrawn at various time intervals for PCA extraction and ^{31}P-NMR analysis. Figure 6 shows that the ATP pool was the first to fully recover, reaching a plateau during the first min. AMP and ADP decreased symmetrically. Glucose 6-P (and also trehalose 6-P, glycerol 3-P, and fructose 6-P), which was not detected in dry lichen, reached the value measured in wet lichens during the two min following rewetting. Interestingly, ru-1,5-DP started to accumulate after a 2–3 min delay corresponding to the time taken for photosynthesis to recover (Fig. 2, trace a2), and reached the level shown in Fig. 3c ca 5 min later. Contrary to this, glcn-6-P decreased to ca one fifth of its initial value during the first 3 min before stabilising. NADP$^+$ was reduced to NADPH from the first min (not shown on the graph). The pool of intermediates involved in membrane lipid syntheses, P-choline and GPC, significantly increased during the first 5 min following rewetting (not shown on the graph).

Conversely, when hydrated, lichens were left drying under natural conditions (outside on a plate), which took ca 30 min, their metabolic profile reverted to that of initially dry thalli. In particular, glcn-6-P started to accumulate and glc-6-P to decrease as soon as the water content of lichens dropped below 30–35% (results not shown). In parallel, NADPH decreased and NADP$^+$ accumulated symmetrically, suggesting that either the cell's requirement for redox power during dehydration was not met and/or that there was a blockage of NADPH production. The accumulation of glcn-6-P suggested that the first NADPH-producing step was stimulated and/or reflected an inhibition of the second NADPH-producing (and glcn-6-P consuming) step. In order to document these hypotheses, we decided to block the pentose phosphate pathway prior to thalli dehydration. To this end, glc-6-P dehydrogenase,

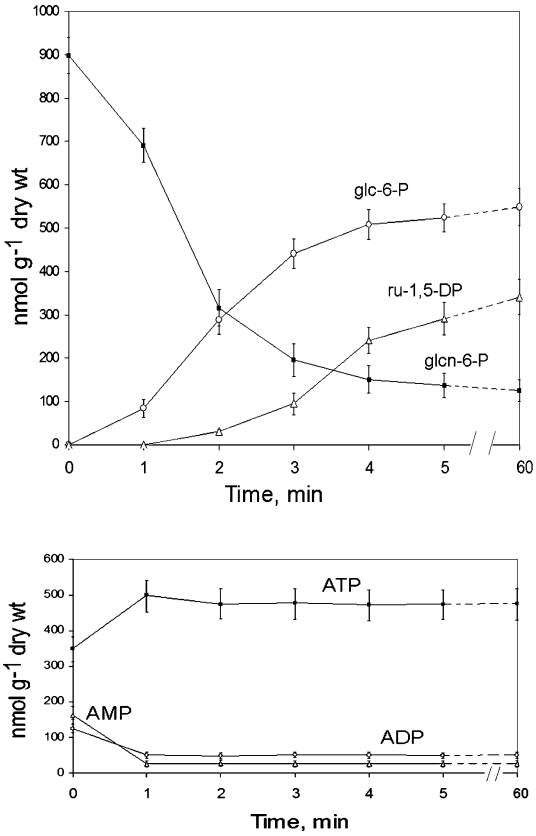

Fig. 6 Time-course evolution of glcn 6-P, glucose 6-P, ribulose 1,5-diphosphate, ATP, ADP, and AMP in *X. elegans* thalli following hydration in the light. At time zero, thalli fragments were incubated in a well aerated liquid medium containing 0.2 mM Mops buffer (pH 6.2), at 20°C. Metabolites were quantified as indicated in Material and methods. Values are means ± SD ($n = 5$)

which converts glc-6-P into glcn-6-P, was inhibited using glucosamine 6-P, a competitive inhibitor of the enzyme (Glaser and Brown 1955). As such, wet thalli were incubated for 1 h in the dark in the presence of 5 mM glucosamine, which was taken up by lichen cells and phosphorylated to N-glc-6-P (Table 2). As previously observed with tobacco cells (Pugin et al. 1997), glcn-6-P and NADPH decreased substantially, and $NADP^+$ increased, whereas glc-6-P remained constant. When these thalli were subsequently dehydrated, NADPH was no longer detected, like in the control dry lichen (Table 1). However, in contrast to control lichen dehydrated in the absence of N-glc, glcn-6-P did not increase (Table 2). In addition, lichens treated with glucosamine showed a marked delay (1–2 min) after rehydration before respiration attained full speed while no delay was observed in control lichens (not shown). Photosynthesis was similarly delayed. These results suggest that the accumulation of glcn-6-P was boosted by the cell dehydrating cell's demand for redox power and that it prompted the recovery of metabolism after rehydration.

Table 2 Modifications of gluconate 6-P, NADPH, and $NADP^+$ induced in *X. elegans* thalli by glucosamine treatment

Metabolite	Wet thalli	Dry thalli
N-glucosamine 6-P	0.38 ± 0.04	0.37 ± 0.04
Glucose 6-P	0.52 ± 0.06	nd
glcn-6-P	0.04 ± 0.01	0.03 ± 0.01
NADPH	0.02 ± 0.01	nd
$NADP^+$	0.07 ± 0.01	0.08 ± 0.01

Lichens were incubated for 1 h in the dark in the presence of 5 mM N-glc (wet thalli) and a fraction of them was subsequently let drying under natural conditions (dry thalli), prior to PCA extraction. Metabolites were identified and quantified as indicated in Table 1. Values are given as µmol g-1 lichen dry weight. Abbreviations as indicated in the legends of Fig. 3; nd, not detected (<0.05 µmol). Values were obtained from a series of independent experiments and are given as mean ± SD ($n = 5$). The levels of *N*-glucosamine 6-P, gluconate 6-P and NADP+ were not statistically different in dry and wet lichens ($P < 0.05$, *t* test)

Discussion

In this study, we present novel findings on the time-course changes of different pools of soluble metabolites in the foliaceous lichen *X. elegans* during hydration/dehydration cycles. Respiration, net photosynthesis, and its metabolite profile were analysed simultaneously during hydration of this lichen. The purpose of this approach was to identify which pools of metabolites were retained in dry lichens, and as such play key roles in their energetic metabolism. Furthermore, we aimed to determine how these metabolites permit the instantaneous restart of respiration and the very efficient recovery of photosynthesis on rewetting.

The first surprise was that dry thalli did not contain hexose phosphates, triose phosphates, and other intermediates of glycolysis, except PGA, which could have been used to fuel respiration during the first seconds following hydration (Fig. 3), in the absence of tricarboxylic cycle intermediates, like pyruvate, malate, succinate, or citrate (Fig. 5). However, in contrast with wet thalli and with most living material examined so far, they contained a very large pool of glcn-6-P (Fig. 3), that can be converted into fructose 6-P and glyceraldehyde 3-P in the pentose phosphate pathway and subsequently contribute to fuel respiration. In fact, the consumption of just half the glcn-6-P pool during the first 3 min following hydration (ca 620 nmol g^{-1} lichen dry weight, Fig. 6) was sufficient to sustain respiration (580 nmol O_2 min^{-1} g^{-1} lichen dry weight). The rest of metabolised glcn-6-P may contribute, via the pentose phosphate pathway, to the recovery of glc-6-P and ru-1,5-DP, which increased symmetrically to the decrease of glcn-6-P. Finally, the replenishment of the ATP pool after hydration was a clear indicator of the recovery of the energetic metabolism. ATP,

which decreased by nearly 30% during dehydration, recovered totally at the expense of AMP and ADP during the first minute following hydration, indicating that adenylate kinase and ATP synthase activities restarted very rapidly (Roberts et al. 1997). Taken together, these results suggest that the stores of glcn-6-P accumulated during dehydration contributed to the rapid and sustained recovery of respiratory and photosynthetic activities in *X. elegans* thalli after rehydration.

The accumulation of glcn-6-P in *X. elegans* during dehydration, when the relative water content of thalli dropped below ca 30%, could have originated from an increase of glucose-6-phosphate dehydrogenase activity, in relation to the production of ROS, as observed in other lichens (Weissman et al. 2005; Kranner and Grill 1994). Indeed, the reducing power (NADPH) is required to limit the potentially damaging effect of the ROS burst due to impaired electron transport chains in water-stressed cells (Rascio and La Rocca 2005). For example, the production of reaction oxygen in the chloroplasts is avoided by reduction of glutathione, via the ascorbate–glutathione cycle (Asada 1994; Foyer et al.1994), which requires NADPH. This mechanism has also been observed in the alpine plant *Soldanella alpina* and in *Pisum sativum* exposed to cold-induced photoinhibition causing glcn-6-P to accumulate (Streb et al. 2003). On the contrary, when the pentose phosphate pathway was blocked after incubating wet thalli in the presence of glucosamine, $NADP^+$ was no longer reduced to NADPH and glcn-6-P did not accumulate during dehydration (Table 2). In addition, a delay in the recovery of respiration and photosynthesis was observed when glucosamie-treated thalli were subsequently rehydrated, as compared to control lichens. These results are consistent with a role of the pentose phosphate pathway in the adaptation of *X. elegans* to hydration/dehydration cycles. Finally, the fact that no other metabolite of the pentose phosphate pathway was detected in dry thalli, in particular ribulose 5-P, suggests that the functioning of 6-phosphogluconate dehydrogenase was blocked before that of glucose-6-phosphate dehydrogenase during cell dehydration.

The protection of many fungi and vascular plants against reactive oxygen species can also involve polyols which constitute alternative metabolic reserves, behave as osmoprotectants, and, like mannitol, are potent quenchers of ROS (Jennings et al. 1998). In this context, we observed that mannitol, when added to a solution of ATP, protected ATP from oxidation during desiccation when exposed to sunlight (result not shown). More generally, polyols, sugars, and other compounds like glutamate, glycine–betaine, etc. stabilise proteins and protect intimate cellular structures against the potentially deleterious effects of dehydration (Hoekstra et al. 2001). Like many other lichens (Vincente and Legaz 1988; Honneger 1991), *X. elegans* contained high amounts of polyols in both photobiont (ribitol) and mycobiont (mannitol and arabitol). These polyol pools did not rapidly change after rehydration. Nevertheless, in accordance with previous results obtained by Farrar (1988), wet thalli collected at dawn contained less arabitol and ribitol than dry thalli (Fig. 5). Arabitol originates from ribitol, a sugar alcohol synthesised by the photobiont, which moves from alga to fungus as demonstrated in *X. aureola* and *X. calcicola* (Richardson and Smith 1968; Lines et al. 1989). Table 1 shows that wet lichens under dark conditions contain lower levels of arabitol than dry lichens. Interestingly, under very long dark periods, arabitol was completely metabolised in wet lichens suggesting that it contributed to sustaining fungal respiration, while mannitol-content remained nearly constant.

The mechanisms of lichen tolerance to dehydration/rehydration cycles include an adaptive response of algal and fungal cells to ROS-induced peroxidation and de-esterification of glycerolipids that permeate membranes, and to mechanical constraints, which lead to cell membrane disruption. Hence, the capacity to rapidly reseal disrupted membranes plays a central role in these mechanisms (McNeil and Steinhardt 1997; McNeil et al. 2003). In this context, our results have shown that, during rehydration, there was a threefold increase in P-choline and GPC nearly doubled (Fig. 5; Table 1). The increase of these two precursors of phosphatidylcholine synthesis (van der Rest et al. 2002) may reflect the synthesis of phosphatidylcholine-rich membrane systems, like the plasma membrane or tonoplast, and thus a role in the maintenance of cell structural integrity. On the contrary, the stability of GPG suggests that thylakoid membranes, which contain most of the cell's phosphatidylglycerol (Joyard et al. 1993) remained intact in the chloroplasts of photobiont during dehydration/rehydration cycles.

In conclusion, our data suggest that the very rapid recovery of *X. elegans* respiration and photosynthetic activities following rehydration was facilitated by the accumulation of stores of glcn-6-P during dehydration and by coordinated events associated with preventing oxidative damage and protecting cell components and structures. Glcn-6-P appeared to accumulate in response to several factors including a need for reducing power in the cell, to limit desiccation-generated ROS and the blockade of glcn-6-P metabolisation. The sizeable pools of polyols present in both phyco- and mycobionts served to protect cell constituents like nucleotides and proteins, and to preserve the integrity of intracellular structures. In lichen thalli, like in other poikilohydric organisms such as seeds, progressive dehydration modifies and finally stops metabolic activities. But, as opposed to seeds where dormancy is advantageous to avoid undesirable germination during transiently-favourable conditions (Bewley 1997), the ability of lichen thalli to restart respiration and photosynthesis without delay permits them to take advantage

of all reviviscence opportunities offered by the presence of both water and light, particularly at low temperatures (Fig. 1). This is the case, for example, when winter sun melts ice to temporarily reveal rocky slopes. In such situations, high net photosynthetic activities achieved at low temperatures will enable synthesis of carbohydrates within minutes following rehydration. Finally, this work highlights Glcn-6-P accumulation during dehydration as a metabolic adaptation of lichens to the anhydrobiotic cycles imposed by the high-mountain climate.

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