Freezing of air-dried lichens ensures that thalli remain healthy for later physiological measurements

Luca Paoli[1,2], *Silvana Munzi*[1], *Tommaso Pisani*[1], *Anna Guttová*[2], *Stefano Loppi*[1*]

[1]Department of Environmental Science, University of Siena, Italy

[2]Institute of Botany, Slovak Academy of Sciences, Slovakia

Abstract

We tested whether the freezing of air-dried lichen thalli alter physiological parameters commonly used as valuable stress markers in laboratory and field ecophysiological studies, namely integrity of cell membranes (measured as electrolyte leakage), assimilation pigments (chlorophyll *a*, chlorophyll *b*, chlorophyll *a*+*b*, total carotenoids content), chlorophyll integrity (OD_{435}/OD_{415}) and chlorophyll *a* fluorescence emission (expressed as F_V/F_M, the potential quantum yield of primary photochemistry and PI_{ABS}, a global indicator of the photosynthetic performance). Thalli of the lichen *Evernia prunastri* (L.) Ach. were air-dried, stored in the freezer at ca. -18°C and analysed after short-term storage (15, 30 and 90 days). These periods are compatible with the needs of a relatively rapid data evaluation in biomonitoring studies. After freezing, the investigated parameters showed that lichens remained healthy and suitable for later physiological measurements without biasing data quality.

Key words: air pollution, cell membranes, chlorophyll, *Evernia prunastri*, performance index

Introduction

Why should storage of lichens be a matter of concern? The reasons are clearly stated by Honegger (2003): in most cases lichenologists have to collect fresh material far away from their laboratories and need to preserve the thalli unaltered. This is particularly true for conservation biologists, in order to transplant viable material (e.g. rare species) for recovery after habitat destruction. Similarly, lab and field work (e.g. experimental studies and transplant experiments) for bioindication purposes may require fresh material collection and safe storage of the samples until someone has time to work on them.

Desiccation and rehydration are part of the natural life style of a lichen (Weissman et al. 2005), since many species spend most of their life in a dry metabolically inactive state and are able to survive for prolonged periods when the thallus water content is below 10% (Rundel 1988). Freezing of air-dried lichen thalli is generally recommended as storage system for subsequent physiological studies (Feige & Jensen 1987). However, lichen responses to the same storage conditions were also found to be species specific (Larson 1989; Honegger 2003).

In this work we tested whether freezing of air-dried thalli of the lichen *Evernia prunastri* (L.) Ach. at -18°C alter specific physiological parameters, namely integrity of cell membranes, assimilation pigments, chlorophyll integrity and chlorophyll *a* fluorescence emission, widely applied as stress markers in laboratory and field ecophysiological studies and considered early bioindicators of air pollution (Boonpragob 2002; Jensen & Kricke 2002; Garty 2002; Paoli & Loppi 2008).

In particular, during recent experiments the above parameters were analysed in *E. prunastri* and contributed to detecting early stress symptoms following exposure to pollutants - either under controlled conditions or in the environment (Munzi et al. 2010; Paoli et al. 2010), to habitat modifications (Paoli et al. 2011) and climatic gradients (Pirintsos et al. 2011).

We focused our attention on short-term storage and specific markers useful in biomonitoring surveys, compatible with the needs to perform fairly rapid analyses during an ecophysiological study.

Materials and Methods

Thalli of *E. prunastri* (length at least 4 cm) were collected from *Prunus spinosa* and *Quercus pubescens* twigs in a remote, rural, hilly area of central Italy (Ancaiano, 11°10'54'' E, 43°17'29" N, Grw; 500 m asl), far from pollution sources. Samples were immediately brought to the laboratory, cleaned with plastic tweezers to remove extraneous material and air-dried up to 48 hours in a climatic-chamber, ensuring standardized pre-conditioning at 15°C, RH 55±5%, photoperiod of 12 hours at 40 μmol m^{-2} s^{-1} photons PAR. Air-dried thalli were then divided in 10 separated batches, each representing a replicate. The material was then put in paper bags, further in plastic bags and then stored at -18°C. Measurements of physiological parameters were carried out prior to freezing (controls) and after 15, 30 and 90 days of freezing.

The integrity of cell membranes was checked by placing a piece of lichen thallus in deionised water and measuring the variation in electrical conductivity (Munzi et al. 2009). In damaged cell membranes, permeability is altered and electrolyte leakage occurs, mainly K+. The peripheral part of the thalli (i.e. ca. 100 mg selected from the outer 2 cm) was rinsed three times in 50 mL deionised water, 5 sec each, to remove dust deposited on the lichen surface. Samples were then soaked in glass bottles with 50 mL of deionised water and shaken for 1 h. Water conductivity was measured before and after sample soaking using a conductimeter (*Basic 30*, *Crison*, Alella, Spain). Thalli were then boiled for 10 min to cause total rupture of cell membranes, and conductivity measured again. Relative conductivity (EC%) was expressed as percent ratio between conductivity after 1 h soaking and after boiling, after accounting for the initial conductivity of deionised water.

Assimilation pigments and chlorophyll degradation in *E. prunastri* were measured as previously described in Pisani et al. (2007). Photosynthetic pigments were extracted using dimethylsulfoxide (DMSO), adding polyvinylpyrrolidone (PVP) and filtering the solution before use. Chlorophyll *a*, chlorophyll *b*, chlorophyll *a*+*b* and total carotenoids were estimated using the equations proposed by Wellburn (1994). *Evernia prunastri* contains usnic and evernic acids and atranorin. Free evernic acids may have deleterious effects on the amount of total chlorophylls (Rapsch & Ascaso 1985) and could degrade chlorophyll during extraction causing phaeophytinization (Brown & Hooker 1977). To remove these substances, before pigment extraction, samples were subjected to six 5-min washings in 3 mL 100% acetone buffered with $CaCO_3$. Lichens were then air-dried for ca. 30 min at room temperature to allow for complete acetone evaporation. Chlorophyll integrity was expressed by the ratio between the absorbance values at 435 and 415 nm (OD_{435}/OD_{415}), as suggested by Ronen & Galun (1984).

Measurements of chlorophyll *a* fluorescence emissions were performed by a plant efficiency analyser (*Handy PEA*, *Hansatech instruments*, Norfolk, UK). Prior to measurements, physiological recovery of the lichens was carried out: to avoid any osmotic stress by air humidity after the freezing, samples were left 30 min in dry ambient conditions. They were subsequently sprayed with water until wet and the excess water was removed by hand-shaking. Samples were then stored at 4°C in the dark for 24 h, ensuring they were wet all along this period. Lichens were handled only in the dark or with a dim green light. After 24 hours samples were prepared for measurements selecting homogeneous marginal parts of the thalli (the outermost 2 cm).

In order to measure each sample, the selected lichen material was dark-adapted with a clip for 10 min to allow full dark adaptation of the photosynthetic pigments. Lichens rested on a foam pad whilst in the clip to minimise damage to the structure. Samples were lightened 1 sec with a saturating 3000 μmol m^{-2} s^{-1} light pulse. Fluorescence emission was recorded for 1 sec; both the dark adaptation period and intensity of saturating light pulse were experimentally determined. Twenty fluorescence emission curves were recorded for each treatment.

Chlorophyll *a* fluorescence emission was analysed by the standard physiological indicator of photosynthetic efficiency F_V/F_M, representing the potential quantum yield of primary photochemistry (Maxwell & Johnson 2000). In addition, the performance index PI_{ABS}, a global indicator of the photosynthetic performance, was calculated to express the overall vitality of the samples:

$PI_{ABS} = RC/ABS \cdot \varphi P_0/(1 - \varphi P_0) \cdot \psi_0/(1 - \psi_0)$

Theory and derivation of the formula can be found in Strasser et al. (2000).

One way ANOVA ($p<0.05$) was run to detect differences among controls before freezing (freshly collected and air-dried samples) and after 15, 30 and 90 days of storage at -18°C.

Results

Results are summarised in Table 1. They show that air-drying of *E. prunastri* and storage in the freezer do not alter integrity of cell membranes, assimilation pigments, chlorophyll integrity (OD_{435}/OD_{415}) and chlorophyll *a* fluorescence emission (F_V/F_M and PI_{ABS}).

The fast fluorescence kinetic typically outlines a transient curve: when the curve is plotted on a log-time axis a sequence of steps, each corresponding to its changing inclination, is apparent (Strasser et al., 2000). In Figure 1, fast fluorescence kinetics of control thalli and samples after 90 days of freezing are shown. The overlapping of the curves suggests that the storage did not alter significantly the kinetics of chlorophyll *a* fluorescence emissions.

Thallus water content of air-dried thalli prior to freezing ranged between 8–11% and remained unchanged along the storage.

Discussion

In general, key factors affecting the viability of lichens during storage are thallus water content, temperature and length of the storage. Freezing of wet thalli or keeping the samples at room temperature can heavily affect lichen physiology, biasing data quality and making the samples useless for bioindication purposes.

On the other hand, several studies reported that air-drying and subsequent freezing would not alter significantly lichen viability for long periods (Larson 1989; Honegger 2003).

Photosynthetic properties of lichen thalli change during desiccation (Hajék et al. 2006), depending on temperature and osmotic stress (Gloser & Gloser 2007) and freezing of wet thalli may lead to mechanical stress to cells due to ice crystal formation.

Larson (1989) investigated the influence of thallus water content during storage and the effects of prolonged freezing in five *Umbilicaria* species stored at high (saturated) and low (air-dried) water content for 1 and 9 months and later on after 10 years. Tolerance to subzero temperatures was species and treatment specific: fully hydrated and frozen thalli generally lost their photosynthetic capacity in the long period and, depending on the species, some variations in maximum photosynthetic rates were observed already in the short-term freezing of dry thalli (Larson 1982). On the other hand, chlorophyll content and chlorophyll integrity ($OD_{435/415}$) significantly decreased in two species out of five in wet thalli, but assimilation pigments did not differ from controls in air-dried thalli (Larson 1989). He estimated water content in thalli prior to freezing near 7%, similar to our thalli.

Yamamoto et al. (1998) evaluated the impact of 3–12 months storage on spore discharge of dry lichen thalli at room temperature, refrigerator (5°C) and freezer (-25°C) and recommended storage in the freezer at -25°C to preserve the viability of the thalli. Honegger (2003) evaluated the impact of storage conditions on the viability of the symbiotic partners in lichens with *Trebouxia* photobionts. After storage at room temperature, both partners of the symbiosis died off within 3 years. Indicators of irreversible damage were discoloration of the thalli, breakage of fungal membrane systems, chlorophyll autofluorescence decrease and incapacity to rehydrate (Honegger 2003). On the contrary, both the mycobiont and the photobiont were viable up to 13 years in

desiccated thalli of *Xanthoria parietina* (L.) Th.Fr. stored at -20°C, their coloration and chlorophyll fluorescence being unchanged. Kappen (1973) showed that freezing air-dried thalli even to very low temperatures and for extended periods of time does not materially damage the symbiotic partners. Gauslaa and Solhaug (1999) performed physiological studies on air-dried *Lobaria pulmonaria* (L.) Hoffm. stored 6 months at -18°C and found out that chlorophyll fluorescence parameters did not change after freezing.
In conclusion, air-drying of *E. prunastri* and storage in the freezer (ca. -18°C) for 3 months ensured that thalli of this epiphytic species remain healthy and suitable for later physiological measurements without biasing data quality.

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Table 1. Physiological parameters in *Evernia prunastri* (control and samples stored in the freezer 15, 30 and 90 days). Average ± SD (n=10): electrical conductivity, electrical conductivity as percentage (EC%), chlorophyll *a*, chlorophyll *b*, chlorophyll *a*+*b*, total carotenoids content (μg/mg dw), chlorophyll integrity (OD_{435}/OD_{415}) and chlorophyll *a* fluorescence indicators (n=20) F_V/F_M and PI_{ABS}.

	Control	15 days	30 days	90 days
Electrical conductivity	2.4 ± 0.8	-	-	3.0 ± 1.0
EC%	8.8 ± 2.9	-	-	10.0 ± 3.3
chlorophyll *a*	0.95 ± 0.22	1.02 ± 0.27	0.89 ± 0.17	0.93 ± 0.18
chlorophyll *b*	0.27 ± 0.07	0.27 ± 0.09	0.23 ± 0.05	0.27 ± 0.04
chlorophyll *a*+*b*	1.22 ± 0.29	1.29 ± 0.36	1.13 ± 0.22	1.21 ± 0.24
carotenoids	0.30 ± 0.05	0.32 ± 0.05	0.29 ± 0.04	0.28 ± 0.05
OD_{435}/OD_{415}	1.20 ± 0.06	1.19 ± 0.11	1.18 ± 0.07	1.23 ± 0.06
F_V/F_M	0.658 ± 0.044	0.632 ± 0.067	0.625 ± 0.073	0.626 ± 0.061
PI_{ABS}	0.147 ± 0.040	0.159 ± 0.067	0.147 ± 0.087	0.171 ± 0.080

Figure 1. Chlorophyll fluorescence kinetics in *Evernia prunastri* (control and samples stored in the freezer for 90 days - average of 20 records).