ELSEVIER

Colloids and Surfaces B: Biointerfaces 00 (2002) 1–9

COLLOIDS AND SURFACES B

www.elsevier.com/locate/colsurfb

Freezing of water bound in lichen thallus as observed by ^{1}H NMR. II. Freezing protection mechanisms in a cosmopolitan lichen *Cladonia mitis* and in Antarctic lichen species at different hydration levels

H. Harańczyk [a,*], J. Grandjean [b], M. Olech [c], M. Michalik [d]

[a] *Institute of Physics, Jagiellonian University, ul. Reymonta 4, 30-059, Cracow, Poland*
[b] *Institut de Chimie B6a, COSM, Universite de Liége, Liege, Belgium*
[c] *Institute of Botany, Jagiellonian University, 30-059, Cracow, Poland*
[d] *Institute of Molecular Biology, Jagiellonian University, 30-059, Cracow ,Poland*

Received 19 June 2001; received in revised form 5 November 2001; accepted 9 January 2002

Abstract

Proton NMR spectra at 300 MHz for dehydrated and hydrated thallus of *Cladonia mitis* Sandst. [= *C. arbuscula* (Wallr.) Flot ssp. Mitis (Sandst.) Ruoss], *Himantormia lugubris* (Hue) Lamb and *Usnea aurantiaco-atra* (Jacq.) Bory were recorded. The temperature was decreased from room temperature down to −45 °C. Pulse length was set to $\pi/2 =$ 8.3 μs, which allowed the observation of tightly bound and loosely bound/or free water fraction, whereas the signal from solid matrix of thallus was reduced. In hydrated thallus a narrow Lorentzian line coming from loosely bound/free water fraction was recorded. For the temperature range between −5 and −20 °C a discontinuous increase in line halfwidths, accompanied by a decrease in area under the peak, was observed. This was attributed to the cooperative freezing of bulk water present in lichen thallus. In dehydrated thallus the NMR line consists of two components: a narrow, Lorentzian one (coming from loosely bound/free water fraction) and a broad line (from water tightly bound in lichen thallus). The overall area under peak remains unchanged down to −5 °C, and then between −5 and −20 °C it continuously decreases due to non-cooperative water immobilisation. As the temperature is decreased, for temperatures above −5 °C, the contribution made by the broad line component increases at the expense of the narrow line component. The mechanism of loosely-to-tightly bound water transfer is, at least partially, responsible for the freeze-protection of thallus in the lichen species investigated. No significant differences between the freeze protecting loosely-to-tightly bound water transfer mechanism of Antarctic lichens and that of cosmopolitan lichens was noticed. © 2002 Published by Elsevier Science B.V.

Keywords: Proton NMR; Microheterogeneous systems; Freezing protection; Lichens; *Cladonia mitis*; *Himantormia lugubris*; *Usnea aurantiaco-atra*

* Corresponding author
E-mail address: hubert@castor.if.uj.edu.pl (H. Harańczyk).

0927-7765/02/$ - see front matter © 2002 Published by Elsevier Science B.V.
PII: S0927-7765(02)00150-9

1. Introduction

Several species of lichens are well adopted to function at extremely low temperature and low hydration level [1]. They recover the life activity after low temperature stress (for some species after the temperature of liquid nitrogen) [2–5] and perform their living functions (the photosynthetic CO_2 uptake) below the water freezing point [1,6–10] and even below the ice heterogeneous nucleation point of lichen thallus fluids [4,11,12].

Lichens are able to take water from snow omitting the liquid phase and the hydration level obtained in this way is sufficiently high to start the photosynthetic activity [5]. They passively take water from the gaseous phase [13–15]. *Himantormia lugubris* and *Cladonia mitis* reversibly dehydrate down to the two-dimensional percolation threshold of water [16,17] and water clustering point [15] which occurs at hydration level lower than needed to stop living processes in thallus [8,18].

The aim of our research was to observe the behaviour of tightly and loosely bound water during slow (in equilibrium) cooling of the thallus from room temperature down to −45 °C. We selected three lichen species; two Antarctic samples: *Himantormia lugubris*, *Usnea aurantiaco-atra* and a cosmopolitan one: *Cladonia mitis*. To examine the dependence of the effectiveness of freeze-protection mechanism on water content, we performed the temperature courses at two hydration levels of thallus, both obtained through hydration from the gaseous phase.

Proton spectra were recorded using the pulse power sufficient to observe both tightly bound water and free water signals ($\pi/2 = 8.3$ μs), whereas the signal from solid matrix of thallus was significantly reduced. Thus, our study is complementary to the temperature dependent measurements in the time domain (free induction decays) using hard pulses ($\pi/2 = 1.1$ μs) [19], and to the experiment involving soft pulses ($\pi/2 = 35$ μs) [20], where solid signal was not observed and the signal from tightly bound water was reduced.

2. Materials and methods

Himantormia lugubris and *Usnea aurantiaco-atra* were collected in King George Island, South Shetlands during the Antarctic summer, whereas *Cladonia mitis* was collected in Northern Sweden, in autumn. The thallus was stored at room temperature and room humidity.

The vitality tests using the fluoresceine biacetate and etidine bromide showed that in *Cladonia mitis* thallus 50–60% of cells reacted positively, in *Himantormia lugubris* 40–50% of cells and in *Usnea aurantiaco-atra* 80%.

Prior to NMR experiments, each type of thallus was divided into two samples. In each case, one was incubated for 48 h over the surface of H_3PO_4 ($p/p_0 = 9\%$) and the other over a supersaturated solution of Na_2SO_4 (93%). Samples were then placed in NMR tubes and gently pressed. NMR tubes were sealed using plastic tube sealings (American Can Company). Tight caps prevent the samples from the change of moisture content during the experiments.

Proton NMR spectra were recorded on a Bruker AM 300 WB spectrometer working at the resonance frequency of 300 MHz. The pulse length was $\pi/2 = 8.3$ μs. The number of scans was 4 for the hydrated sample and 16 for the dry one. The relaxation delay was 3s between scans. The number of data points recorded per spectrum was 4 K; all spectra were processed without line broadening. After averaging the RSS value was between 1 and 1.6 per data point.

The temperature was stabilised in gaseous nitrogen flow with the accuracy of about 1 K.

Line deconvolutions were performed using about 1200 data points per spectrum. Based on SIMPLEX procedure, the Bruker LINESIM program was used. For dehydrated samples, several different functions were tested to fit the spectrum properly; namely, the superposition of Lorentzian and Gaussian line, two Lorentzians and two Gaussians. The narrow line component was well fitted using Lorentzian line in all samples investigated, whereas the broad line is fitted with sufficiently good quality using either Gaussian or Lorentzian function. This agrees with measurements in time-domain, where tightly bound water

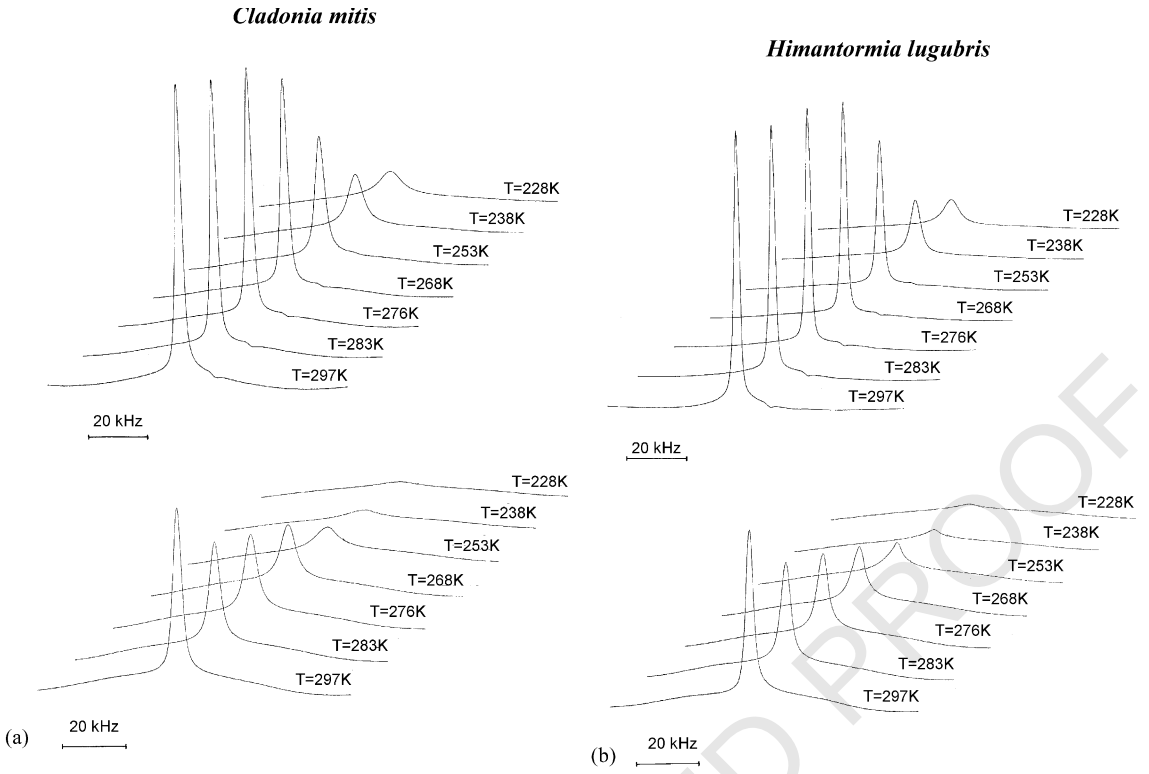

Fig. 1. Proton NMR spectrum recorded at 300 MHz for the thallus as a function of temperature; (a) *Cladonia mitis*, the sample hydrated in $p/p_0 = 93\%$, upper plot, the sample hydrated in $p/p_0 = 9\%$, lower plot; (b) *Himantormia lugubris*, the sample hydrated in $p/p_0 = 93\%$, upper plot, the sample hydrated in $p/p_0 = 9\%$, lower plot; (c) *Usnea aurantiaco-atra*, the sample hydrated in $p/p_0 = 93\%$, upper plot, the sample hydrated in $p/p_0 = 9\%$, lower plot.

signal is fitted by an exponential function [15,19]. The fits assuming individual Lorentzian line shape were good (RSS value between 1 and 1.6 per data point).

3. Results

As the solid matrix of the thallus relaxes with $T_2^* \approx 16$ μs (time after which Gaussian signal decreases to its $1/e$ value) and the tightly bound water fraction relaxes exponentially with $T_2^* = 60$–100 μs [15], the π/2 pulse length of 8.3 μs allowed us to detect both mobile and tightly bound fraction of water bound to the lichen thallus, whereas the signal coming from the solid matrix of thallus was significantly reduced.

The spectra of dehydrated thalli consisted of two line components: broad and narrow. For hydrated samples the broad line component was hardly visible and only *Cladonia mitis* revealed the pronounced contribution of broad line (Fig. 1). Fitted line widths and areas under peak are presented in Table 1, Table 2, and Table 3 for *Cladonia mitis*, *Himantormia lugubris*, and *Usnea aurantiaco-atra*, respectively.

The narrow line component may be connected with the free water or water loosely bound in the lichen thallus. The halfwidths of the broad line component was about 32–39 kHz. Except for

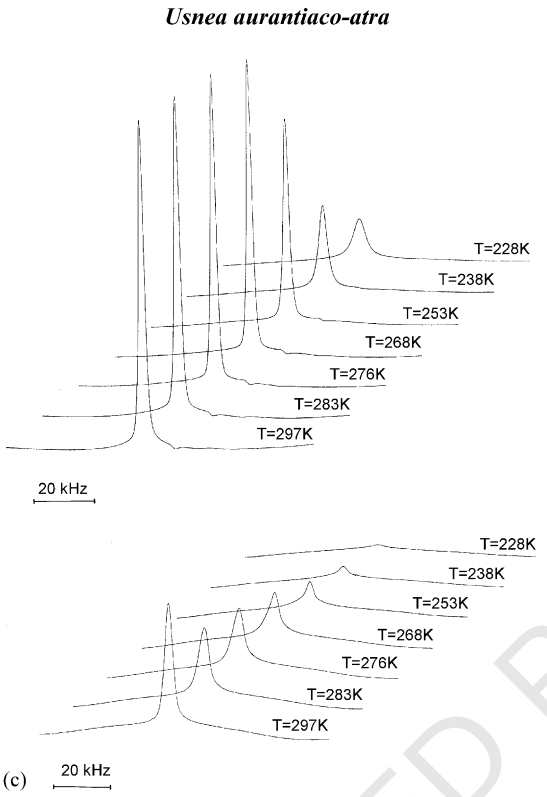

Fig. 1 (*Continued*)

Cladonia mitis, it was not possible to deconvolute the composite spectrum for hydrated sample, because the contribution of loosely bound/free water pool overwhelms the tightly bound water fraction. For sake of consistency, we present the total areas under peaks and narrow peak positions.

Fig. 2a, c, and d shows the halfwidths of the proton line for hydrated samples of investigated lichens. For the temperature range above −20 °C the line halfwidth is constant and equals 6.78 ppm for *Cladonia mitis*, 5.39 ppm for *Himantormia lugubris* and 5.20 ppm for *Usnea aurantiaco-atra*. As the sample is macro-heterogeneous, the measured value of linewidth is significantly broadened both by the non-uniformity of the effective magnetic field, B_{eff}, and by the magnetic susceptibility non-uniformity. For lower temperature the proton line broadens. This is attributed to freezing of the loosely bound water fraction at −12 °C. This interpretation is consistent with high power proton relaxometry results in *Cladonia mitis* [19,21].

For hydrated *Cladonia mitis* the spectrum may be deconvoluted into the sum of two components (Fig. 2b). For the temperatures above −20 °C the average Lorentzian line halfwidth (6.45 ± 0.24 ppm) obtained from the deconvolutions is only slightly different from the corresponding value of the halfwidth (Fig. 2a) obtained in the single component analysis. The broad Gaussian line is observed with the halfwidth of about 100 ppm for the whole range of investigated temperatures. For the temperatures below −20 °C it significantly broadens, which means that the structural change in loosely bound water fraction affects also the tightly bound water protons (Fig. 2b).

For hydrated samples of lichen thalli the total area under peak remains constant (or slightly decreases) with decreasing temperature down to $t = -10$ °C (Fig. 3), whereas, for further decreas-

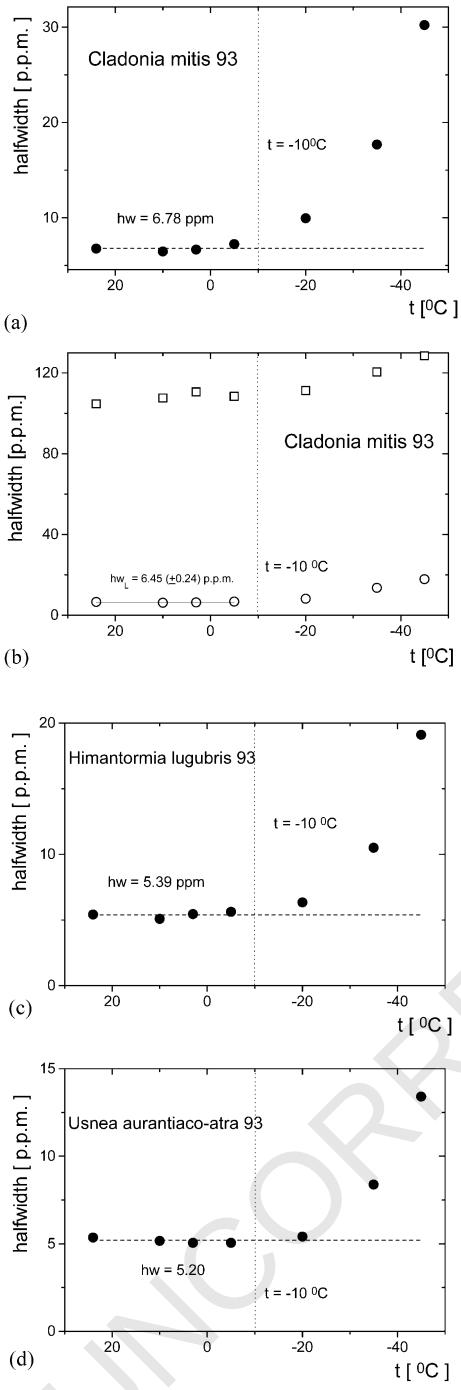

Fig. 2. The temperature dependence of the proton NMR linewidth recorded at 300 MHz for thallus hydrated in $p/p_0 = 93\%$. (a) *Cladonia mitis*; (b) *Cladonia mitis* after decomposition of line into broad and narrow components; (c) *Himantormia lugubris*; (d) *Usnea aurantiaco-atra*.

ing temperature the total area under peak rapidly decreases. At $t = -40$ °C the total area under line is 50% of its initial value. The decrease in area under line is correlated with the line halfwidth increase, suggesting that the free water pool at low temperatures may be transferred to a phase which differs from the free water observed at higher temperature.

Fig. 4 shows the temperature dependence of the area under broad and narrow component of the proton NMR line for the dehydrated thallus of the investigated lichens. For *Himantormia lugubris* and for *Usnea aurantiaco-atra*, the total signal remains almost constant for temperatures above $t = -10$ °C, whereas for lower temperatures it smoothly decreases with decreasing temperatures. For *Cladonia mitis* the signal continuously decreases with decreasing temperature for the whole temperature range, which is the effect implied by the desorption isotherm requirements [22].

The behaviour with temperature of the tightly bound and loosely bound water signal intensities are found to be similar for Antarctic and cosmopolitan lichen species. As the temperature is decreased, the loosely bound water signal significantly decreases. This decrease is compensated for by the increase in the tightly bound water signal.

The decrease in mobile line component is almost linear with temperature for *Himantormia lugubris* and for *Usnea aurantiaco-atra*, whereas for *Cladonia mitis* its magnitude stabilises at 8.7% of the total signal for the temperature $t = -20$ °C and lower temperatures. None of the samples show any discontinuities or breaks in the slope of temperature dependence of area under peak at $t = -35$ °C.

Our results suggest that the process of the transfer of loosely water signal to the tightly bound water pool above $t = -10$ °C occurs continuously with the temperature decrease.

4. Discussion

In hydrated thalli, below −10 °C a rapid decrease in the total area under peak was observed, which is caused by the freezing of loosely bound water. This discontinuity was observed at

Table 1
The temperature course for *Cladonia mitis* hydrated at $p/p_0 = 9\%$ (a) and at $p/p0 = 93\%$ (b)

a	Temperature		Area under peak		
	t (°C)	T (K)	(a.u.)	Broad (%)	Narrow (%)
	24	297	227.4	61.5	38.5
	10	283	215.5	77.2	22.8
	3	276	203.2	78.6	21.4
	−5	268	184.2	82.4	17.6
	−20	253	152.8	90.0	10.0
	−35	238	104.2	91.8	8.8
	−45	228	82.3	92.0	8.0
b	Temperature		Halfwidth		Area under peak (a.u.)
	t (°C)	T (K)	(Hz)	(ppm)	
	24	297	2028.7	6.76	240.6
	10	283	1938.0	6.46	236.1
	3	276	1996.8	6.65	237.8
	−5	268	2171.5	7.23	232.2
	−20	253	2983.5	9.95	212.8
	−35	238	5307.7	17.69	167.0
	−45	228	9073.8	30.22	139.5

−12 °C by high power proton relaxometry in the thallus of *Cladonia mitis* [19,21].

A break in the slope of the temperature dependence of proton spin-lattice relaxation time at $t =$

Table 2
The temperature course for *Himantormia lugubris* hydrated at $p/p_0 = 9\%$ (a) and at $p/p_0 = 93\%$ (b)

a	Temperature		Area under peak		
	t (°C)	T (K)	(a.u.)	Broad (%)	Narrow (%)
	24	297	224.53	70.0	30.0
	10	283	226.20	75.0	25.0
	3	276	219.88	82.3	17.7
	−5	268	199.92	82.7	17.3
	−20	253	168.49	90.4	9.6
	−35	238	121.95	97.3	2.7
	−45	228	111.47	99.2	0.8
b	Temperature		Halfwidth		Area under peak (a.u.)
	t (°C)	T (K)	(Hz)	(ppm)	
	24	297	1622.5	5.41	137.0
	10	283	1525.6	5.08	130.2
	3	276	1634.8	5.45	126.6
	−5	268	1684.4	5.61	119.2
	−20	253	1901.8	6.33	94.7
	−35	238	3152.6	10.5	65.4
	−45	228	5721.7	19.1	53.3

Table 3
The temperature course for *Usnea aurantiaco-atra* hydrated at $p/p_0 = 9\%$ (a)and at $p/p_0 = 93\%$ (b)

a	Temperature		Area under peak		
	t (°C)	T (K)	(a.u.)	Broad (%)	Narrow(%)
	24	297	195.56	64.2	35.8
	10	283	189.39	76.2	23.8
	3	276	189.54	79.6	20.4
	−5	268	180.91	84.3	15.7
	−20	253	141.39	92.2	7.8
	−35	238	114.83	96.4	3.6
	−45	228	93.92	99.1	0.9
b	Temperature		Halfwidth		Area under peak (a.u.)
	t (°C)	T (K)	(Hz)	(ppm)	
	24	297	1606.0	5.35	145.32
	10	283	1548.3	5.16	144.88
	3	276	1516.7	5.05	148.79
	−5	268	1517.6	5.05	144.42
	−20	253	1625.1	5.41	132.21
	−35	238	2515.4	8.38	97.75
	−45	228	4024.2	13.4	79.34

−35 °C was observed in the dehydrated thallus of *Cladonia mitis* [21]. As this effect was observed only in dehydrated samples, it suggested that it is caused by the structural changes in the thallus solid matrix. The present experiments did not reveal any discontinuities in the area under peak at $t = -35$ °C for dehydrated thallus. As these experiments did not record the signal from solid matrix of thallus, but from bound water only, the above supports the hypothesis that the effect at $t = -35$ °C occurs in solid matrix of lichen thallus and, moreover, the tightly bound water layer is not involved in this process [21].

The change in the slope, at $t = -45$ °C, of the proton spin-lattice relaxation time versus temperature plot was attributed to subtle structural change in water tightly bound in thallus of *Cladonia mitis* [21]. This effect was not observed here as the present measurements were not performed below $t = -45$ °C.

The variety of data available on the ability of lichen to recover CO_2 uptake after cooling the thallus down to low temperatures, taken together with direct recording of photosynthetic CO_2 uptake at low temperatures represents convincing evidence that an extraordinarily effective freezing protection mechanism exists in the thallus. This mechanism enables it to recover living activity even after freezing in liquid nitrogen and must preserve the thallus during ice crystallite growth which otherwise might destroy thallus microstructures. The physical basis for such a freezing protection mechanism has not been established so far. However, observations of Antarctic arthropodes and variety of plants [23–29] suggest that polyhydric alcohols and simple sugars may act as cryoprotectants. Glycerol present in aqueous medium of wheat photosynthetic membrane preparations protects the system from the ice crystallite formation [30,31]. Although glucose and fructose were not found in *Evernia esorediosa* (Müll. Arg.) Du Rietz, in *Ramalina subbreviuscula* Asah., and in *R. sublitoralis* Ash., in samples collected in nature, and the significant content of monosugars was only forced by external osmotic conditions, the polyols (ribitol, mannitol, arabitol), as essential metabolites, were present in thalli on constant level (up to 3.4% w/w of arabitol in *R. subbreviuscula*) [32] and may act as cryoprotective agents in thallus. Using ^{13}C-NMR, Chapman et al.

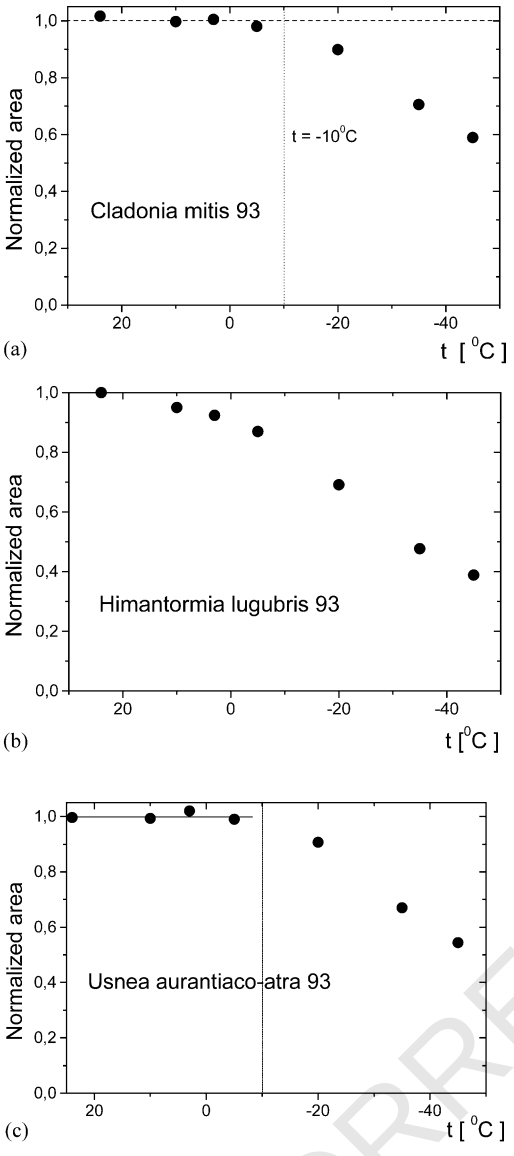

Fig. 3. The normalised area under proton NMR line for hydrated thallus of *Cladonia mitis* (a), *Himantormia lugubris* (b) and *Usnea aurantiaco-atra* (c) versus decreased temperature.

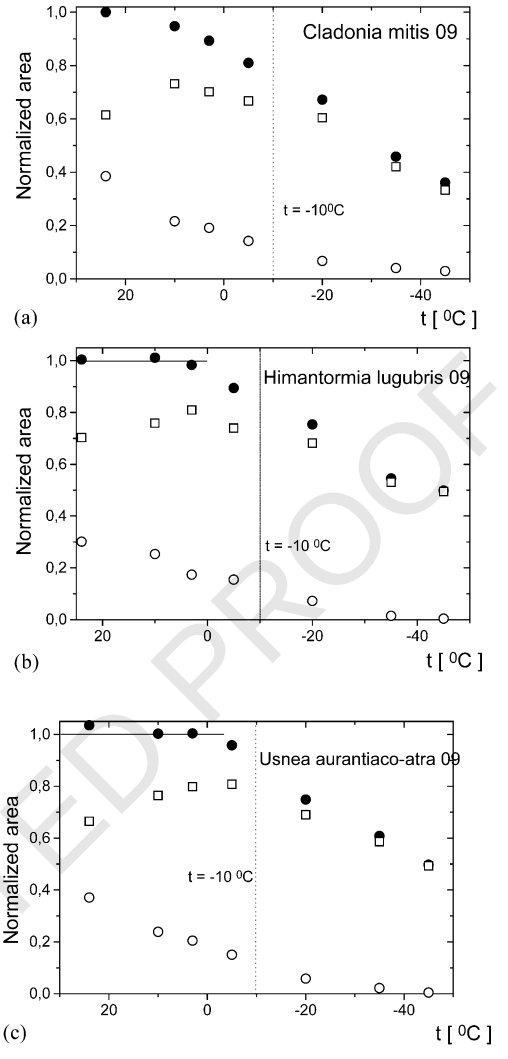

Fig. 4. The temperature dependence of normalised area under peak in NMR spectrum for dehydrated thallus of *Cladonia mitis* (a), *Himantormia lugubris* (b) and *Usnea aurantiaco-atra* (c); black dots—total area, open circles—narrow line component and open squares—broad line component.

[33] identified polyols and sugars, and quantified their abundance, in several Antarctic lichens. Polyol content varied between 17 mg g^{-1} for *Candelariella hallettenensis* (Murray) Ørsted [= *C. flava* (Dodge & Baker) Castello & Nimis] and 65 mg g^{-1} for *Usnea antarctica* Du Rietz, with dominating contribution from arabitol, mannitol and ribitol, whereas sorbitol was not detected. Sugar content was approximately one order of magnitude smaller than the content of polyols. In Antarctic bryophytes the repeated freeze-thaw cycles (up to $n = 16$) increase rates of carbohydrate

loss, which is not significantly correlated with the DSC-detected freezing temperature of tissue [34].

The cryoprotective action of polyols is based on blocking the formation of ice crystallites by steric mismatch of hydrogen bonds which may be formed between them and water. Although the overall concentration of polyols detected in the thallus is not sufficient to promote significant decrease of freezing point of cellular aqueous medium, if they are localized mainly in intracellular spaces, they may contribute to the frost protection mechanism in lichens.

We did not observe the increase of the total liquid proton signal upon cooling the thallus, as it should be observed if liquid polyols were rapidly produced by dissolution of the solid matrix of dry biological system [35]. We propose that the mechanism of water transfer from loosely bound (freezable) to tightly bound (non-freezable) pool may play a significant role in freeze protection of lichen thallus. The tightly bound water may produce an 'insulating' layer surrounding the ice crystallites as they are probably formed in inner spaces of lichen thallus.

In lichen cellular fluids ice nucleation is observed [3,11,12,36,37] at temperatures well above the lowest temperature at which the photosynthetic activity occurs. If this is the case for the lichen thallus, ice nucleation in this system is not necessarily a destructive process.

It is thought that extracellular water freezing occurs in freezing tolerant plants and the lethal process of intracellular formation of hexagonal ice crystal is avoided [38]. Most likely, in lichen, the thallus acts not only to protect it from ice crystallite growth in intra-cellular spaces during freezing, which otherwise might destroy the thallus microstructure, but also to promote ice crystallite formation in extra-cellular spaces, which minimizes the effect of potentially destructive intracellular ice crystallite formation. The effect of the stimulation of the non-lethal growth of ice crystallites in extra-cellular space may result in accumulation of the increased water amount in form of ice crystallites (in extra-cellular space), which can be advantageous for lichens living in sites with permanent moisture deficit.

Acknowledgements

This work was supported by the partnership contract between Jagiellonian University and University of Liège.

References

[1] L. Kappen, Antarctic Sci. 1 (1989) 31–34.
[2] O.L. Lange, L. Kappen, Photosynthesis of lichens from Antarctica, in: G.A. Llano (Ed.), Antarctic Terrestial Biology Antarctic Research Series, vol. 20, American Geographical Union, Washington, 1972, pp. 83–95.
[3] T.H. Nash, III, L. Kappen, R. Lösch, D.W. Larson, U. Matthes-Sears, Flora 179 (1987) 241–251.
[4] T.H. Nash, III, L. Kappen, R. Lösch, U. Matthes-Sears, D.W. Larson, Cold resistance of lichens. Progress and problems in lichenology in the eighties, Bibl. Lichenol. 25 (1987) 313–317.
[5] L. Kappen, Arctic 46 (1993) 297–302.
[6] O.L. Lange, H. Metzner, Naturwissenschaften 52 (1965) 191–192.
[7] E.D. Schulze, O.L. Lange, Flora 158 (1968) 180–184 (Abt. B).
[8] L. Kappen, Polar Biol. 1 (1983) 249–255.
[9] L. Kappen, E.I. Friedmann, Polar Biol. 1 (1983) 227–232.
[10] B. Schroeter, T.G.A. Green, L. Kappen, R.D. Seppelt, Crypt. Bot. 4 (1994) 233–241.
[11] T.L. Kieft, V. Ahmadjian, Lichenologist 21 (1989) 355–362.
[12] B. Schroeter, Ch. Scheidegger, New Phytol. 131 (1995) 273–285.
[13] L. Kappen, Water relations and net photosynthesis of Usnea. A comparison between Usnea fasciata (Maritime Antarctic) and Usnea sulphurea (Continental Antarctic), in: D.H. Brown (Ed.), Lichen Physiology and Cell Biology, Plenum Press, New York and London, 1985, pp. 41–56.
[14] L. Kappen, J. Redon, Flora 179 (1987) 215–229.
[15] H. Harańczyk, S. Gaździński, M. Olech, New Phytol. 138 (1998) 191–202.
[16] D. Stauffer, A. Aharony, Introduction to percolation theory, Taylor&Francis, London, 1991.
[17] H. Harańczyk, M. Wnęk, S. Gaździński, J. Grandjean, M. Olech ,1998. Dehydration below percolation threshold and freezing protection mechanism in lichens as observed by NMR and electric conductivity, 62.Physikertagung 1998 der Deutschen Physikalischen Gesellschaft, Regensburg, Hauptvortrag AOE 2.3, in preparation.
[18] L. Kappen, M. Sommerkorn, B. Schroeter, Lichenologist 27 (1995) 531–545.
[19] H. Harańczyk, S. Gaździński, M. Olech, Freezing Protection mechanism in *Cladonia mitis* as observed by proton magnetic relaxation, in: New Aspects in Cryptogamic Research. Contribution in Honour of Ludger Kappen.