

## Differential effects of spring reacclimation and deacclimation on cell membranes of Norway spruce seedlings

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### Abstract

Two-year-old seedlings of Norway spruce (*Picea abies*) during spring deacclimation were subjected to controlled reacclimation by exposure to low temperatures of 4/–3°C (day/night) in a cold room. The highest increase in freezing tolerance (by 7°C) was observed after 12 d of low temperature exposure, when shoot water potential ( $\Psi_{w\text{ shoot}}$ ) decreased to 0.64 MPa. The process of reacclimation was accompanied by an increase in the phospholipid content of needle cell membranes. This increase applied to total (PL) and individual phospholipids: phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidic acid (PA). After being exposed to the low temperature for 18 d, the seedlings were moved into the open air. This caused deacclimation, with an increase in  $\Psi_{w\text{ shoot}}$  to –0.36 MPa and a decrease in the total phospholipid content and freezing tolerance of the needles. Significant correlations were observed between freezing tolerance, the membrane permeability (MP) of the needles and the phospholipid content,  $\Psi_{w\text{ shoot}}$  and water content of the needles. The results show that during spring deacclimation, Norway spruce seedlings can be subjected to reacclimation, which is reflected in the phospholipid content, the biophysical changes of the membranes, and the freezing tolerance of the seedlings. During both spring deacclimation and reacclimation, water content in the needles plays a critical role in the cold tolerance of spruce seedlings.

**Keywords:** freezing tolerance, membrane permeability, phospholipids, *Picea abies*, deacclimation, reacclimation

### Introduction

Plant tolerance to freezing is genetically conditioned to a large extent, but it is also dynamic, changing seasonally [1,2]. Environmental factors, such as temperature, water deficit, and light, regulate the cellular level of expression of genes associated with plant dormancy, cold hardiness, and development. After growth cessation, mainly under the influence of short days, tree buds begin endodormancy [3], which is transformed into ecodormancy, mainly due to low temperatures. At the end of winter, day length and mean air temperatures increase, while freezing tolerance declines, gradually releasing ecodormancy [4]. This release starts the process of deacclimation in trees. There is little information about the early stages of spring deacclimation/reacclimation. The reaction to exposure deacclimation, warm temperatures and light is dependent upon the species. Woody plants exhibit two forms of deacclimation: “active” and “passive”. Active deacclimation occurs in spring

and is associated with the resumption of growth. Passive deacclimation results from the exposure of fully acclimated plants in mid-winter to 5°C or higher temperatures. Some researchers suggest that the reversal process, reacclimation, can be observed during deacclimation in spring [3]. During reacclimation, the return to tolerance levels similar to those observed in mid-winter is impossible, as was observed in Scots pine [4,5]. Reacclimation potential decreases with increasing deacclimation duration. In trees of boreal forests, the process of acclimation in autumn is slower (weeks to months) than spring deacclimation (days to a week). In tree seedlings grown under controlled conditions, both acclimation and deacclimation can be successfully initiated. The strongest effects of acclimation in evergreen conifers are observed in autumn [2,6,7]. Our first study on multiple acclimation and deacclimation of current-year Norway spruce shoots was carried out in mid-winter [8]. During deep dormancy, spruce shoots were successively deacclimated and again acclimated by subjecting them first to temperatures of 22°C and 15°C and long days, followed by a temperature of 1°C and short days, reaching a level of freezing tolerance similar to the initial level [5,9]. However, successful full deacclimation/reacclimation was possible only until the end of February [9]. The major sites of biochemical transformations, as well as of freezing injuries in the needles of coniferous trees, are cytoplasmic membranes [10]. The composition of phospholipids and polyunsaturated fatty acids affects the biophysical state of membranes (phase structure), and this determines membrane permeability to ions [6,11]. In autumn, with the progress of acclimation, membrane permeability to water increases.

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In their studies, Siminowitch et al. [12], Sensor and Beck [13,14], and Iivonen et al. [15] demonstrated a correlation between the level of plant acclimation and the phospholipid content of the tissues. Needles of acclimated seedlings of *Pinus sylvestris* had higher phospholipid content than non-acclimated ones [16]. These investigations have been considerably extended by other studies [17–19]. These studies observed a clear relationship between the process of acclimation and the concentrations of phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). There are some signals that appear in the coniferous species *Picea glauca*, *Pinus banksiana* and *Pinus contorta* during plant deacclimation in spring, including increasing day length and air temperature and a gradual decrease in the concentrations of phospholipids, polyunsaturated fatty acids, sugars and proteins, which accumulated during acclimation in autumn [20,21].

In many parts of the northern hemisphere, cold snaps in late winter and early spring, when the process of deacclimation begins, are particularly dangerous for trees [22]. Extreme frost injury in late winter after warm periods and after late spring frosts has often been recorded in Poland when the temperature drops below  $-8.0^{\circ}\text{C}$  in April [23]. In late winter and early spring, temperature variation (day/night) is accompanied by lowered humidity, which leads to a strong water deficit in the photosynthetic organs of evergreen trees.

The aim of this study was to investigate the ability of spruce seedlings during spring deacclimation to respond to changes in cold hardiness during reacclimation caused by temperature variations simulating the late winter and early spring periods. The study focused on how the disturbance of deacclimation by the process of induced reacclimation is reflected in the water relation, individual phospholipid content, free fatty acid content and the biophysical properties of cytoplasmic membranes in spruce needles.

## Material and methods

### Plant material

Two-year-old Norway spruce seedlings [*Picea abies* (L.) Karst.] of the submontane provenance Węgierska Górk (49°34'N, 19°10'E, 750 m a.s.l.) were obtained in March from a forest nursery in the Konstantynowo Forest District (near Poznań, Poland). The seedlings were transferred from the nursery to plastic boxes containing a perlite: peat mixture (1:5, v:v) and were then split into blocks and replicates. One portion of the seedlings was subjected to a reacclimation treatment at 4/–3°C day/night for 18 d (April 10–28). These seedlings were stored in a cold room at a relative humidity of 52% with day length increasing from 14 to 16 hours, simulating the outdoors, at  $50\ \mu\text{mol m}^{-2}\text{ s}^{-1}$  of photon flux density. To minimize soil freezing during the cold nights, the pots were placed in insulated boxes. On the 18th day of the experiment, the reacclimated plants were transferred outdoors and kept with the control seedlings (Fig. 1b). During the reacclimation treatment, the control seedlings of the same provenance were kept outdoors under the canopy of trees. The entire experiment lasted from April 10 until May 22.

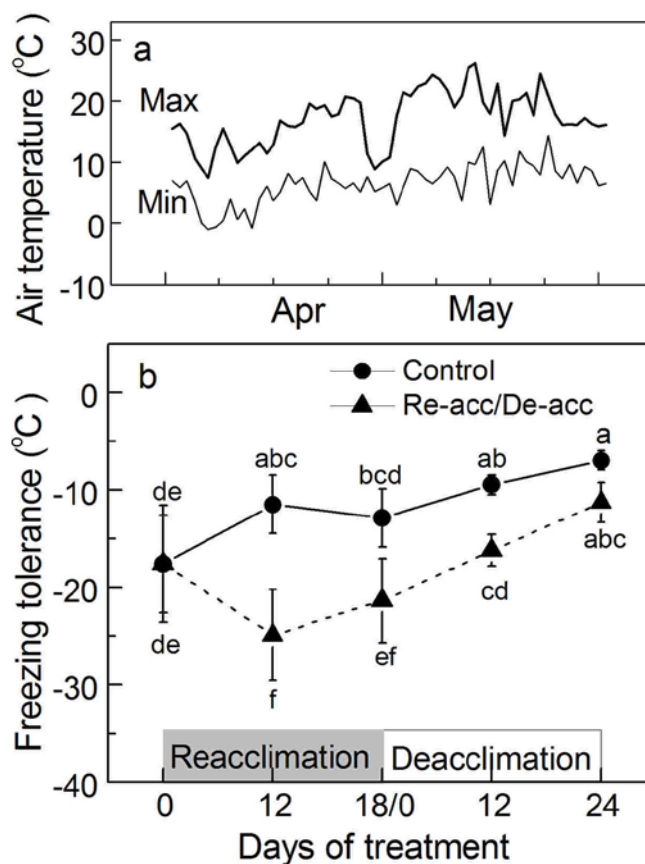
### Determination of freezing tolerance

Changes in the freezing tolerance of the needles during reacclimation cycles were measured using the method of

Pukacki and Pukacka [10]. Needles from current-year spruce shoots were placed in test tubes in a computer-controlled freezer (Binder MK 53, Germany). After 0.5 h equilibration at  $0^{\circ}\text{C}$ , the temperature was decreased at a rate of  $0.05^{\circ}\text{C min}^{-1}$ . The needle samples were withdrawn at  $5^{\circ}\text{C}$  intervals and allowed to thaw at  $3^{\circ}\text{C}$  overnight. After freezing, the needles were thawed at a rate of  $0.07^{\circ}\text{C min}^{-1}$ . The needles were then placed into distilled water ( $0.1\ \text{g ml}^{-1}$ ). The percentage of ion leakage from the samples was determined by measuring its electrical conductivity of the solution after incubation for 24 h at room temperature. The conductivity was measured using a temperature-compensating conductivity meter (CPC-551, Elmetron, Poland). To measure total conductivity, the samples were killed by freezing at  $-60^{\circ}\text{C}$  for 80 min, after which they were re-extracted for 24 h at room temperature in the original solution. The cold hardiness of needles was defined as the lethal temperature ( $LT_{50}$ ) and was calculated as the temperature producing 50% ion leakage.

### Membrane permeability (MP)

The level of MP to ions was expressed as the conductivity of the leakage from the needles. Samples (1 g each) of spruce needles were soaked in distilled water (1:10, w:v). The conductivity of the solution was measured after incubation for 48 h, according to Zwiazek and Shay [24]. The specific ion leakage from the needles in siemens (S) per gram fresh weight ( $\mu\text{S g}^{-1}\text{ FW}$ ) was used as an indicator of membrane permeability.



**Fig. 1** The maximum and minimum air temperatures (a) in Kórnik ( $52^{\circ}15'\text{N}$ ,  $17^{\circ}04'\text{E}$ ), measured approximately 200 m away from the experimental site (70 m altitude), and the effects of reacclimation (Re-acc) and deacclimation (De-acc) on the freezing tolerance of needles ( $LT_{50}$ ; b). Mean values  $\pm\text{SD}$  ( $n = 5$ ). Significant differences (according to the Duncan test,  $P < 0.05$ ) are indicated by different letters.

### Measurement of water relation

The water potentials of the shoot ( $\Psi_{w \text{ shoot}}$ ) were measured using a pressure bomb (PMS) instrument (Corvalis, USA) according to a previously described method [25].

The measurements were repeated every 3 d. When  $\Psi_{w \text{ shoot}}$  decreased to  $-0.64$  MPa (which occurred after 12 d of reacclimation) and  $-0.72$  MPa (after 18 d of reacclimation), biochemical analyses were performed that measured the following: phospholipids,  $\alpha$ -tocopherol, sterols, free fatty acids, malondialdehyde, and freezing tolerance. After the transfer of the seedlings to field conditions, when  $\Psi_{w \text{ shoot}}$  increased to  $-0.50$  MPa (after 12 d of deacclimation) and  $-0.36$  MPa (after 24 d of deacclimation), the above analyses were repeated.

The water content (WC) of the needles was expressed in terms of the percentage of fresh weight using the following equation:  $WC = (FW - DW)/DW$ ; DW – dry weight. The samples were dried at  $74^\circ\text{C}$  for 72 h.

### Lipid peroxidation

The level of lipid peroxidation in the needles was measured as malondialdehyde (MDA) formation and determined by means of reaction with thiobarbituric acid (TBA), according to Heath and Packer [26]. The needles were ground in a cold mortar using 5% trichloroacetic acid (TCA). The homogenate was centrifuged at  $12000\text{ g}$  for 15 min. A  $1000\text{ }\mu\text{l}$  aliquot of 2 ml 0.5% (w/v) TBARS containing 20% TCA was added to  $200\text{ }\mu\text{l}$  aliquots of supernatant. The mixture was heated at  $95^\circ\text{C}$  for 30 min, and the reaction was stopped by placing the reaction tubes in an ice bath. The thiobarbituric acid-malondialdehyde (TBA-MDA) complex absorption was measured at 532 nm and corrected for non-specific turbidity at 600 nm. The concentration of MDA was calculated using a molar extinction coefficient of  $155\text{ nM}^{-1}\text{ cm}^{-1}$  [26]. Each data point represents the mean  $\pm$ SD of five measurements.

### Separation and identification of phospholipids

The samples (5 g each) of needles were ground to powder in liquid nitrogen, and the lipids were extracted with chloroform:methanol (2:1, v:v), according to Juaneda and Requelin [27] and Nichols et al. [28]. The separation of polar and neutral lipids was performed using a SEP-PAK Silica cartridge (Waters As, No. 51900) [29]. The phospholipids were fractionated by TLC on silica gel 60 plates in chloroform:methanol:acetic acid:water (85:15:10:3.5, v/v) [30]. The classes of phospholipids were identified and determined as inorganic Pi, according to the method of Pukacki and Kamińska-Rożek [31]. The quantities of particular phospholipids were expressed as  $\mu\text{g P mg}^{-1}$  lipids.

### Free fatty acids (FFA), $\alpha$ -tocopherol and sterols

Free fatty acids and  $\alpha$ -tocopherol were assayed in the total fraction of the lipid extract according to Pukacki and Kamińska-Rożek [31] after silylation by N-O-bis-trimethylsilyltrifluoroacetimide (BSTFA) with heptadecanoic acid (C17) and  $\alpha$ -cholestin as internal standards. The samples were separated by gas chromatography (Hewlett Packard 5890) with a capillary column SPB-1, an injector and flame ionization detector temperature of  $280^\circ\text{C}$ , a split injector, and a carrier gas (helium) linear flow rate of  $2\text{ cm}^3\text{ min}^{-1}$ . FFA,  $\alpha$ -tocopherol and sterols were identified by comparing the retention times with those of authentic standards obtained from Sigma-Aldrich. The operating conditions were as follows: column temperature  $160\text{--}280^\circ\text{C}$ ; rate  $4^\circ\text{C min}^{-1}$ .

### Statistical analysis

The data are presented as the means  $\pm$ SD. The significance of the differences between the control and stressed plants were assessed on the basis of the analysis of one-way variance (ANOVA) and linear regression for each parameter. Significant differences between the means were determined using Duncan's multiple range test, and the significance levels are indicated as  $P < 0.05$ . Analyses of variance and correlations between variables were performed using the statistical software Statgraphics Plus. For all correlation analyses, the percentages were transformed (arcsin) so that the data conformed to parametric test assumptions.

## Results

### Changes in freezing tolerance, MP and water relations during reacclimation/deacclimation

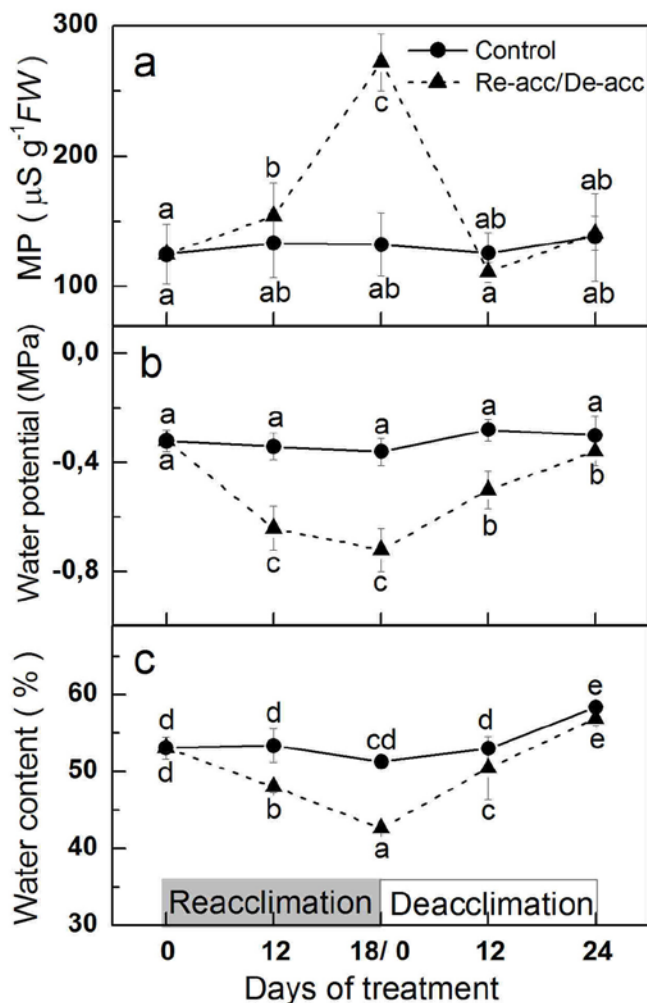
At the beginning of this study, the spruce seedlings were in the spring deacclimation period. The maximum and minimum temperatures of March were above zero (Fig. 1a). The freezing tolerance ( $LT_{50}$ ) of 1-year-old needles was  $-17.6^\circ\text{C}$ . After 18 d of cold reacclimation, the freezing tolerance of the needles significantly increased from  $-17.6^\circ\text{C}$  to  $-22^\circ\text{C}$  (Fig. 1b), while in the control seedlings that were kept outdoors, it decreased to  $-11.5^\circ\text{C}$  and then continued to decrease to  $-7.0^\circ\text{C}$  until May. The increase of the freezing tolerance after 18 d of the seedling needles under reacclimation was accompanied by a significant increase of MP (Fig. 2a), and decrease of the water potential of the shoots ( $\Psi_{w \text{ shoot}}$ ) from  $-0.32$  MPa to  $-0.64$  MPa and  $-0.72$  MPa, respectively (Fig. 2b), and a decrease of the water content in the needles from 53% to 48% and 43%, respectively (Fig. 2c). After 18 d of reacclimation, the needles reached the highest level of membrane permeability to ions, which was increased two-fold (from 132 to  $272\text{ }\mu\text{S g}^{-1}\text{ FW}$ ) relative to the control seedlings that were kept outdoors (Fig. 2a).

During deacclimation, when the seedlings were returned to the outdoors the values of the water potential of the shoots and the water content, as well as membrane permeability gradually returned to the values of the control samples. However, the freezing tolerance of the seedlings was still higher than that of the non-reacclimated controls. The degree of freezing tolerance in the reacclimation seedlings after 24 days of deacclimation was  $4^\circ\text{C}$  higher relative to the control seedlings kept outdoors (Fig. 1b). Here, a significant positive correlation between the  $LT_{50}$  of the seedlings during deacclimation and the average daily air temperature ( $r = 0.535$ ,  $P < 0.03$ ) was observed using linear regression.

### Phospholipids, $\alpha$ -tocopherol, sterols, and lipid peroxidation

To examine the changes in the structure of membranes in the needles of spruce seedlings subjected to reacclimation, analyses of the amounts of total and individual phospholipids were performed. After 12 days of reacclimation, when the water potential of shoots decreased to  $-0.64$  MPa (Fig. 2b) in needles, a significant increase in the total phospholipids was observed: 11% compared to the control ( $P < 0.05$ ; Tab. 1). The levels of particular phospholipids (PC, PG, PE and PA) also increased (77%, 54%, 30% and 87%, respectively; Tab. 2). After 18 days of reacclimation, a continued increase in the phospholipids was observed but to a lesser extent (9% compared to the control seedlings kept outdoors). During deacclimation, decreases in the total and individual phospholipid contents were observed





**Fig. 2** Effects of reaccclimation (Re-acc) and deaccclimation (De-acc) in spring on the following: membrane permeability of current-year needles of spruce seedlings (a), shoot water potential (b), and water content in needles (c). Mean values  $\pm$ SD ( $n = 5$ ). Significant differences (according to the Duncan test,  $P < 0.05$ ) are indicated by different letters. MP – membrane permeability.

simultaneously with an increase in the water potential of the seedling shoots (Fig. 2b, Tab. 1, Tab. 2). A significant, negative correlation was found between the membrane permeability to ions and the water content in the needles and the water potential in the shoots ( $\Psi_{w \text{ shoot}}$ ):  $r = -0.66$  ( $P < 0.0003$ ) and  $r = -0.55$  ( $P < 0.004$ ), respectively (Tab. 3). As well a significant negative correlation was found between the freezing tolerance of the needles and the total phospholipid contents:  $r = -0.71$ ,  $-0.74$ ,  $-0.59$  and  $-0.74$  for PC, PG, PE and PA, respectively ( $P < 0.001$ ; Tab. 3). However, a positive correlation was found between the contents of individual phospholipids and membrane permeability:  $r = 0.49$ ,  $0.53$  and  $0.58$  for PC, PG, and PA, respectively ( $P < 0.01$ ; Tab. 3). During the reaccclimation of spruce seedlings, the contents of free fatty acids (FFA) did not change significantly; only an increase in  $\alpha$ -tocopherol was noted after 18 d of reaccclimation (from  $2.04$  to  $2.72 \text{ ng g}^{-1} \text{ DW}$ ) and in the content of sterols after 12 days of deaccclimation (from  $2.64$  to  $3.80 \text{ nmol mg}^{-1} \text{ DW}$ ; Tab. 1). Lipid peroxidation, as measured by malondialdehyde (MDA) levels, during the treatment was generally consistent ( $1.19$ – $1.48 \text{ nmol mg}^{-1} \text{ DW}$ ), irrespective of the reaccclimation/deaccclimation of the seedling needles.

## Discussion

In late winter post-dormancy, plants can be easily de-hardened [32]. Alternatively, repeated decreases in air temperature at night to several degrees below  $0^\circ\text{C}$  can lead to reaccclimation or can delay the deaccclimation of plants, as shown in the present study. In our earlier studies, which were conducted in autumn and mid-winter, 3-year-old Norway spruce seedlings treated with a constant temperature of  $-3^\circ\text{C}$  did not increase their freezing tolerance. Only at much lower temperatures ( $-10^\circ\text{C}$ ) did their freezing tolerance increase by  $6^\circ\text{C}$ . In the present study, which was conducted in early spring, the temperature cycle  $4/-3^\circ\text{C}$ , day/night, which simulated the conditions occurring in spring, had a positive effect on the reaccclimation of Norway spruce seedlings. During 12 d of cyclically changing temperatures, seedlings showed an increase in freezing tolerance of  $7^\circ\text{C}$  and were able to withstand a temperature of  $-25^\circ\text{C}$ . Simultaneously, a decrease in the shoot water potential was observed, which could evoke many reactions leading to the increased resistance of cells to frost injury [2].

**Tab. 1** The effects of reaccclimation ( $4/-3^\circ\text{C}$ , day/night) and deaccclimation on total phospholipids, free fatty acids,  $\alpha$ -tocopherol, sterols and malondialdehyde content of needles of Norway spruce seedlings in spring.

Re-acc/de-acc (days)	Treatment	PL ( $\mu\text{g mg}^{-1}$ lipids)	FFA ( $\text{ng g}^{-1} \text{ DW}$ )	$\alpha$ -tocopherol ( $\text{ng g}^{-1} \text{ DW}$ )	Sterols ( $\text{ng g}^{-1} \text{ DW}$ )	MDA ( $\text{nmol mg}^{-1} \text{ DW}$ )
0	Control	$6.35 \pm 0.88$	$3.91 \pm 1.10$	$2.04 \pm 0.52$	$3.90 \pm 0.38$	$1.50 \pm 0.20$
12	Control	$6.31 \pm 0.98$	$2.98 \pm 0.65$	$1.90 \pm 0.29$	$3.10 \pm 0.69$	$1.07 \pm 0.20$
	Re-acc	$8.05 \pm 0.87^*$	$4.36 \pm 0.99$	$1.92 \pm 0.12$	$3.29 \pm 0.50$	$1.26 \pm 0.10$
18	Control	$6.93 \pm 0.92$	$3.55 \pm 0.80$	$2.40 \pm 0.40$	$3.72 \pm 0.70$	$0.93 \pm 0.07$
	Re-acc	$7.58 \pm 0.62$	$2.98 \pm 0.50$	$2.72 \pm 0.20$	$3.15 \pm 1.22$	$1.19 \pm 0.10$
12	Control	$6.33 \pm 0.69$	$3.12 \pm 0.00$	$2.05 \pm 0.01$	$2.64 \pm 0.01$	$1.15 \pm 0.10$
	De-acc	$6.60 \pm 0.91$	$2.54 \pm 1.29$	$2.25 \pm 0.58$	$3.80 \pm 0.75^*$	$1.18 \pm 0.30$
24	Control	$5.02 \pm 1.06$	$3.73 \pm 1.89$	$2.81 \pm 0.15$	$3.87 \pm 0.84$	$1.48 \pm 0.30$
	De-acc	$3.78 \pm 0.80^*$	$3.20 \pm 1.81$	$2.24 \pm 0.52$	$3.08 \pm 0.56$	$1.28 \pm 0.30$

Values are means  $\pm$ SD ( $n = 5$ ). Asterisks represent significant differences between treatments in analysis of variance.  $^* P < 0.05$ . De-acc – deaccclimation; FFA – free fatty acids; MDA – malondialdehyde; PL – phospholipids; Re-acc – reaccclimation.

**Tab. 2** Effects of reacclimation (4/−3°C, day/night) and deacclimation on individual phospholipids content of needles of Norway spruce seedlings in spring.

Re-acc/de-acc (days)	Treatment	PI	PC	PG	PE	PA
		(μg mg <sup>−1</sup> lipids)				
0	Control	0.37 ± 0.06	2.19 ± 0.22	1.21 ± 0.12	1.24 ± 0.19	0.58 ± 0.11
12	Control	0.75 ± 0.20	1.53 ± 0.49	0.74 ± 0.14	1.02 ± 0.23	0.63 ± 0.16
	Re-acc	0.89 ± 0.12	2.72 ± 0.38**	1.14 ± 0.17*	1.33 ± 0.19**	1.18 ± 0.12**
18	Control	0.41 ± 0.11	2.35 ± 0.28	0.93 ± 0.29	0.90 ± 0.25	1.02 ± 0.13
	Re-acc	0.35 ± 0.03	2.79 ± 0.25	1.69 ± 0.24**	1.12 ± 0.19	1.29 ± 0.16*
12	Control	0.37 ± 0.09	2.09 ± 0.22	0.75 ± 0.07	0.74 ± 0.05	0.75 ± 0.14
	De-acc	0.35 ± 0.07	2.09 ± 0.30	1.16 ± 0.20*	0.80 ± 0.20	0.83 ± 0.16
24	Control	0.22 ± 0.08	1.35 ± 0.26	0.62 ± 0.08	0.55 ± 0.14	0.39 ± 0.09
	De-acc	0.19 ± 0.05	0.97 ± 0.12	0.37 ± 0.12*	0.32 ± 0.05	0.22 ± 0.04

Values are means ± SD ( $n = 5$ ). Asterisks represent significant differences between treatments in analysis of variance as follows: \*  $P < 0.05$ ; \*\*  $P < 0.01$ . De-acc – deacclimation; FFA – free fatty acids; MDA – malondialdehyde; PA – phosphatidic acid; PC – phosphatidylcholine; PE – phosphatidylethanolamine; PG – phosphatidylglycerol; PI – phosphatidylinositol; Re-acc – reacclimation.

The freezing tolerance of the spruce needles was strongly related to water content during the reacclimation/deacclimation development. The spruce seedlings subjected to reacclimation at  $\Psi_{w\text{ shoot}} = -0.64$  MPa (after 12 d) showed an increase in freezing tolerance. After reacclimation, during the deacclimation of seedlings in the open air, as the mean daily air temperature, day length and  $\Psi_{w\text{ shoot}}$  increased, the freezing tolerance of the spruce seedlings decreased. After deacclimation (24 d) when  $\Psi_{w\text{ shoot}}$  of the seedlings increased to 0.36 MPa, the freezing tolerance of the seedlings that were previously subjected to reacclimation reached −11.3°C, while in the control seedlings that were kept outdoors, the freezing tolerance reached only −7.0°C. The extent and rate of deacclimation depends not only on the magnitude of the increase in temperature but also on the duration of exposure [2]. The plants subjected to reacclimation followed by spring deacclimation, proceeded more slowly in the de-hardening process, and their freezing tolerance, even after 24 days, was 4.3°C higher than that of the control seedlings kept outdoors. This suggests that reacclimation enables the maintenance of higher cold freezing tolerance during deacclimation, which is observed in late spring.

The association of acclimation and spring deacclimation with the lipid composition of cytoplasmic membranes was

observed by Yoshida and Uemura [18] and Zwiazek et al. [21]. From previous studies, much more is known about the correlation between plant acclimation in autumn than the correlation between spring deacclimation and the content or composition of phospholipids [16]. In isolated thylakoids of *P. sylvestris* needles, a two-fold increase of galactolipids and sulfolipids was observed during autumn, while MGDG (mono-galactosyldiacylglyceride) decreased during early winter and DGDG (digalactosyldiacylglyceride) and sulfolipids remained high during winter, decreasing to low levels in spring [33]. In *Populus* in autumn, a correlation between an increase in freezing resistance and an increase in phospholipid content (mainly PC and PE) was observed by Yoshida and Sakai [34]. In our study, we paid special attention to changes in membrane phospholipids during spring reacclimation/deacclimation, which preceded bud opening. A significant correlation was observed between the freezing tolerance of the seedlings and PL content ( $r = -0.71$ ,  $P = 0.0002$ ). The same trend was visible between needle freezing hardness and PC content of membranes. In the needles of Norway spruce seedlings, along with a decline in  $\Psi_{w\text{ shoot}}$  to −0.64 MPa (after 12 d of reacclimation), the PL content increased by 28% and the levels of individual phospholipids (PC, PG, PE and PA) increased from 30% to 87%. Changes in the lipid content of membranes in plants at low temperatures as well as disturbances in cell ion metabolism may lead to the activation of lipolytic enzymes, such as phospholipase D [35], which is active even at −20°C and causes PC degradation to generate phosphatidic acid and a free headgroup [15]. Wang et al. [36] detected the activity of phospholipases A and D during acclimation. In this study, a significant increase in PC and PA levels in the seedlings subjected to reacclimation was observed after only 12 days of treatment (when  $\Psi_{w\text{ shoot}} = -0.64$  MPa). The increase in PA may indicate an increase in the degradation of PC and also in the activity of phospholipase D [37]. However, the conditions of reacclimation to which they were exposed affected PC accumulation to a larger extent than is possible due to enzymatic degradation [37].

During deacclimation, along with the increase in  $\Psi_{w\text{ shoot}}$ , a decrease in the phospholipid content of the needles was observed. The acclimation of *Salix* phloem parenchyma and spruce needles was associated with metabolic changes in lipids [3,38]. In general,

**Tab. 3** The correlation coefficients ( $r$ ) values for the freezing tolerance ( $TK_{50}$ ), membranes permeability (MP) and water potential ( $\Psi_{w\text{ shoot}}$ ), water content (WC), total phospholipids (PL), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidic acid (PA) content.

Parameters	$TK_{50}$		MP	
	$r$	$P$ -value	$r$	$P$ -value
$\Psi_{w\text{ shoot}}$	0.580	0.02	−0.554	0.004
WC	0.665	0.0003	−0.665	0.0003
PL	−0.715	0.0001	0.371	0.06
PC	−0.747	0.0009	0.493	0.01
PG	−0.593	0.0001	0.531	0.006
PA	−0.743	0.0001	0.585	0.002

Significance level ( $P < 0.01$ ) of values are shaded.

membrane lipid changes are largely a reversal of those observed during autumnal acclimation. A tendency for such changes in membrane lipids in spring can be explained by the decomposition of phospholipids into simpler compounds, followed by the use of these compounds for the synthesis of important metabolites during plant growth initiation [20]. Sometimes, the freezing stress caused by frost desiccation leads to phospholipid degradation (peroxidation) [39,40]. Fatty acid peroxidation is significantly limited by the cell's antioxidant system, initially by low-molecular-weight compounds, mainly glutathione and ascorbate [18,40,41]. Changes in  $\alpha$ -tocopherol levels have not previously been measured during the reacclimation/deacclimation of *Picea* needles. Our analyses did not detect peroxidation, and no effect on the levels of free fatty acids, malondialdehyde or  $\alpha$ -tocopherol was observed during the reacclimation/deacclimation experiment. Thus, the increase in the MP to ions was caused by changes in phospholipid content and most likely by phase changes in the membrane structure [11,39,40]. The MP to ions and water is affected by the composition and content of phospholipids (mainly PC) and their fatty acids, chiefly linolenic acid (18:3) [42,43]. In this study, significant correlations were observed between the MP to ions and the concentrations of three phospholipids: PC, PG and PA. It is interesting that changes in MP are reversible in the reacclimation/deacclimation cycle. Membranes permeability is also affected by sterols [44], which are important components involved in membrane stabilization. In spruce seedlings, no such relationship was observed, as the  $\Psi_{w\text{ shoot}}$  decreased during reacclimation, while sterol content did not change. However, during the increase in  $\Psi_{w\text{ shoot}}$  to  $-0.50$  MPa (during the first days of deacclimation), the sterol content increased relative to the control. It has been suggested that cold hardness is a function of membrane lipid fluidity and/or the transition temperatures of the solid and gel phases.

Our research showed that reacclimation caused a decrease in the water content of the seedling needles and in the  $\Psi_{w\text{ shoot}}$  of the seedlings. In this phase state, the MP to ions increased almost 2-fold compared to control seedlings. This phenomenon depends on the deficit of water vapor compressibility in extracellular spaces and on MP. In this study, negative correlations were observed between the MP to ions and both the water content of the needles and  $\Psi_{w\text{ shoot}}$ . Complete rehydration of the tissues was observed at the end of the deacclimation period. Water conditions are important factors that determine the acclimation and cold hardness of woody plants [2,45]. Tissue moisture can also impact hardness transitions via mechanisms distinct from the effect tissue moisture on extracellular freezing. Needle dehydration significantly decreases the temperature of ice nucleation in extracellular spaces. Additionally, an increase in MP during acclimation and reacclimation is favorable for spruce needles, as it facilitates the leaking of water out of the cytoplasm and protects plants against dangerous intracellular crystallization [21]. If the snow cover in winter is absent, the frost period is prolonged and the air humidity is low, allowing the dangerous process of frost desiccation to take place [22], which degrades the membrane structure. However, as shown by our earlier research [18] and that of other authors [7,19], cytoplasmic membranes are not always injury under such conditions. According to Christersson and Fircks [22], the severity of the injury depends to a large extent on the species and the progress of acclimation.

Because the seedlings subjected to reacclimation increased their freezing tolerance by several degrees and  $\Psi_{w\text{ shoot}}$  reached  $-0.64$  MPa despite the gradual increase in day length (14 to 16 hours), it can be assumed that the reacclimation was caused

mainly by the low temperature of  $-3^{\circ}\text{C}$ . Welling et al. [45], who studied downy birch during spring deacclimation, showed that the dormancy release is conditioned mainly by temperature and, less strongly, by photoperiod. In autumn, inverse relations are observed. Christersson [46] reports that in seedlings of *P. sylvestris* in autumn, short days were more effective than low temperatures.

Earlier research by Welling et al. [47] on hybrid cuttings (*Populus tremula*  $\times$  *P. tremuloides* Michx.) with a gene (phytochrome A) that blocked reactions of growth inhibition by short days, showed that in autumn, short days and low temperature independently induce cold acclimation. Thus, in species of the genera *Pinus* and *Picea* of boreal coniferous forests, different mechanisms can function in autumn and spring to induce the processes of acclimation and deacclimation. Differences in acclimation and deacclimation kinetics may be related to divergent energy requirements. Acclimation involves changes in structure and function, necessitating large amounts of energy. Deacclimation, however, may be a relatively less energy-intensive process [2]. In this study, the seedlings of *P. abies* were obtained from a submontane population (altitude 750 m a.s.l.) and initiated growth in spring several days later than seedlings originating from lowlands [48]. Our results supplement the current knowledge of this phenomenon, as these results showed that spruce seedlings during spring deacclimation have a potential for reacclimation, which predisposes them to delays in spring growth initiation.

It can be concluded that the processes of cold reacclimation of *P. abies* seedlings in spring are induced mainly by low temperature and are not strongly related to photoperiod. These processes are associated with cold-induced biochemical and biophysical membrane alterations, particularly with changing concentrations of phospholipids in membranes and membrane permeability to ions in needles, which may affect the state of the water conditions in tissues as well as freezing tolerance. The potential of evergreen trees to undergo cold reacclimation in spring under the influence of late spring frosts is favorable, as it enables those species to initiate growth and development without frost damage during cold snaps.

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## Authors' contributions

The following declarations about authors' contributions to the research have been made: planned the experiments and supervised biochemical analyses, contributed to data interpretation and prepared the manuscript: PMP; performed chemical and biochemical determinations: EKR.

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