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Membrane properties of Norway spruce cells during long-term freezing stress

Abstract

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Low-temperature desiccation induces several physical and compositional changes in the microsomal membranes isolated from primordial shoots of Norway spruce (*Picea abies* (L.) Karst). Exposure to – 18°C for 40 days in the winter, causes an increase in plasmalemma permeability. The phospholipid (PL), protein and thiol group (-SH) content of microsomal membranes isolated from stressed tissues significantly changes.

Freeze-desiccation stress in which the primordial tissue looses 20% of water, leads to decrease in the amounts of phosphatidylcholine, phosphatidylinositol and phosphatidic acid. There is a positive correlation between PL and tissue water content, and both of parameters were correlated with the viability of tissues. Low-temperature desiccation stress leads to progressive decrease of the total microsomal protein and non-protein sulfhydryl groups content. The results suggest that membrane injury by long-term freeze-desiccation stress of Norway spruce primordial tissues is mediated by structural and biochemical factors.

Additional key words: desiccation, freezing, stress, membranes, injury, phospholipids, primordial shoots.

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INTRODUCTION

Freezing injury is influenced by the site, rate, and extent of ice formation. Under natural conditions ice formation is initiated in woody plants between – 1° to – 4°C (Ashworth 1993). During extracellular freezing, ice first forms in the dilute apoplastic solution and water gradient is established between the extracellular ice crystal and the intracellular water. Under these conditions

liqud water moves from the cell to the extracellular ice, causing the cells to desiccate. Water moves from the inside to the outside of the cell because the vapor pressure of extracellular ice is lower than the vapour pressure of vacuolar and cytoplasmic water at the same temperatures (Steponkus et al. 1992). This process depends on the freezing temperature, on the freeze-period and on the concentration of cell sap determining the dehydration stress on the plant cell. Dehydration of a cell caused by extracellular freezing has long been regarded as a withdrawal of water due to an osmotic gradient between cell fluid and the outside medium concentrated by ice formation. Levitt (1980) pointed out that some plants resist freezing injury by either tolerating or avoiding cellular dehydration. If freezing is mainly intracellular, ice forms *in situ* and water molecules do not move through plasma membrane which therefore cannot be acting as a barrier to ice formation. Thus a clear knowledge of the water status and membrane propertis within plant tissue is important to an understanding of how plant organs tolerate the extracellular freezing. Parallelism between low temperature and drought stress was reported in crop plants.

Cell membranes are major sites influenced both by dehydration, freezing stress (Levitt 1980, Yoshida and Uemura 1990) and ice encasement stress (Hetherington et al. 1988). Recent investigations have shown that changes in membrane composition ascribed to low temperature adjustment could also be caused by water stress (Pukacki 1992, 1994). A change in the physical state of membrane lipids from a flexible liquid crystalline state to a solid gel structure has been proposed as one of the primary responses leading to low temperature-induced and dehydration-induced injury (Kendall et al. 1985, Steponkus et al. 1992). Membrane lipids may also directly modulate the activity and functions of membrane proteins (Carruthers and Melchior 1986). The present study examines changes in the physical and compositional properties of cellular membranes of spruce tissues after exposure to long-term extracellular ice freezing stress.

MATERIALS AND METHODS

One-year-old shoots of Norway spruce (*Picea abies* (L.) Karst), were collected during the winter, from trees growing on an experimental area of the Institute of Dendrology in Kórnik (Giertych 1970).

Desiccation stress

The 5-8 cm long shoots were desiccated in open (fast stress) and/or closed (slow stress) preserve jars and then placed for 6 weeks at −18°C (cold chamber). Moisture content were calculated from the difference between the fresh and dry weights of shoot primordia.

Calorimetry

Temperature measurements of ice formation in buds were carried out using Omega Engineering, Inc., Stamford, high resolution WB-AAI-B8 cards, and differential thermal analysis (DTA) as previously described (Pukacki 1985, Pukacki and McKersie 1990, Pukacki et al. 1991). The samples were frozen from 0°C to −50°C at a rate of 8°C/h with data points every 20 s. Data analysis was done on an IBM-PC AT using Omega Scientific software.

Membrane preparation

Using chilled mortar and pestle 1-2 g of spruce tissue samples were ground in 10 or 15 ml buffer composed as described by Yoshida et al. (1983). The homogenate was centrifuged at 10,000 g for 20 min to remove organelles. Microsomal membranes were prepared from the supernatant by centrifugation at 130,000 g for 70 min (Pukacki et al. 1991).

Chemical composition of microsomal membranes

Phospholipid content and phospholipid classes after separation by one-dimensional TLC were detected as *Pi* according to the method of Ames (1966) as described by Pukacka (1989). The membrane protein content was measured according to Bradford (1976). Total non-protein-SH content was assayed by the procedure of Sedlak and Lindsay (1968) in a microsomal pellet using glutathione as the standard. After homogenization of the microsomal pellet the homogenate was directly deproteinized by incubation in the boiling water bath for 10 min and further centrifuged at 20,000 g for 15 min (0°C).

Plasma membrane permeability

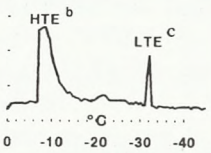
Electrolyte leakage was used as an indirect measure of membrane permeability, a method based on the procedure of Dexter et al. (1932) as described in Pukacki and Pukacka (1987), was used.

RESULTS

Differential thermal analysis (DTA) demonstrated that two distinct freezing events occurred when buds of spruce were frozen (Pukacki 1985, 1989) (Table 1). The first, generally referred to as the high temperature exotherm (HTE), apparently corresponds to extraorgan freezing of water that is outside the primordia shoots. This freezing event does not appear to be the simultaneously

Table 1
Effect of low-temperature desiccation at −18°C on plasmalemma injury and water supercooling in Norway spruce bud tissues.

Water content (%)	Plasmalemma injury (%)[a]	Differential thermal analysis (DTA)
0.68	10.5±0.3	
0.63	22.7±0.3	
0.60	21.1±1.0	
0.55	41.3±0.8	
0.50	49.0±3.5	
0.45	62.1±3.7	



a) Plasmalemma injury calculated according to the formula of Pearce and Willison (1985).
b) HTE – high temperature exotherm.
c) LTE – low temperature exotherm.

injurious and is often indicated on DTA plots as occurring between −7°C and −11°C (Table 1). It is not representative of what occurs in field conditions (Pukacki 1989, Ashworth 1993). The exposure of Norway spruce (*Picea abies* (L.) Karst) buds to fast low-temperature drying (−18°C) leads to a progressive loss of viability of the primordial tissue, as shown by the tetrazolium test, and to the loss of plasmalemma semipermeability, detected in this study as increased ion leakage (Fig. 1, Table 1). These symptoms were initiated after 10

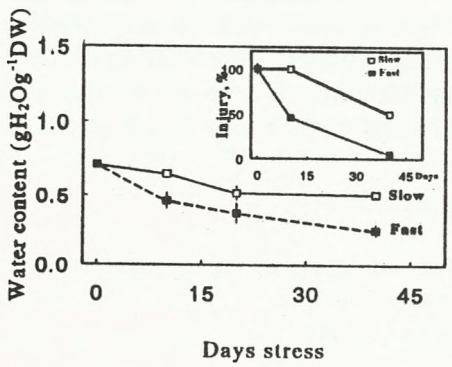


Fig. 1. Effect of long-term low-temperature drying rate (fast and slow), on the moisture contents and injury of Norway spruce primordial bud tissues. Results are means ±SD from three independent measurements. Injury of primordial cells was checked by the tetrazolium test.

days in fast low-temperature drying state. Membrane permeability measurements suggest that plasmalemma was altered by the extracellular or extraorgan ice-dehydration stress. The compositional studies were, however, made on the microsomal fraction. Membrane proteins exhibited significant modifications in

response to the freeze-dehydration stress (Fig. 2) and as is indicated by the phospholipid/protein ratio (Fig. 2). This degradation occurred when the tissue was subjected to the fast drying stress but not during slow drying at low-temperature. The protein and thiol groups level in membrane isolated from fast dried primordial shoots decreased 50% and 40% respectively (Fig. 2). However, the decrease in the thiol group content was initiated earlier when water was reduced about 20%, and protein decrease only after at 40% water loss.

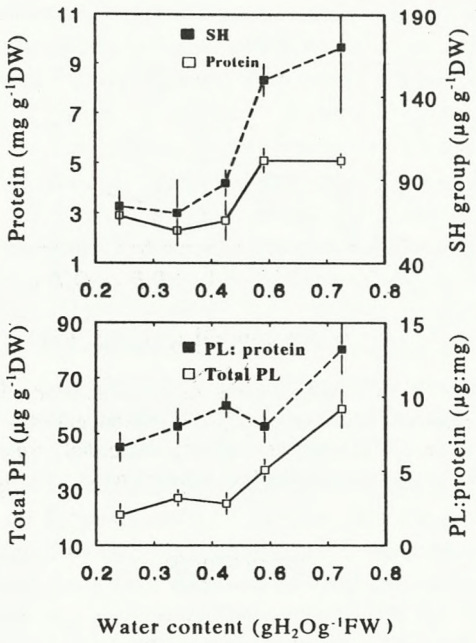


Fig. 2. Effect of low-temperature desiccation stress on non-protein-SH groups content, protein content, total phospholipid content, PL and PL: protein ratio in microsomal membranes of spruce shoot primordial tissues. Results are means ±SD from three independent measurements.

A large decrease in membrane phospholipid content for primordial shoots in spruce occurred both during slow and fast drying, and correlated significantly with the decrease of water content ($r=0.93$) and of viability. Chemical analysis of membrane lipid component revealed changes in total phospholipid and in main phospholipid classes. The phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatic acid (PA) content isolated from the microsomal fraction revealed significant decrease during long-term freezing-desiccation stress (Fig. 3).

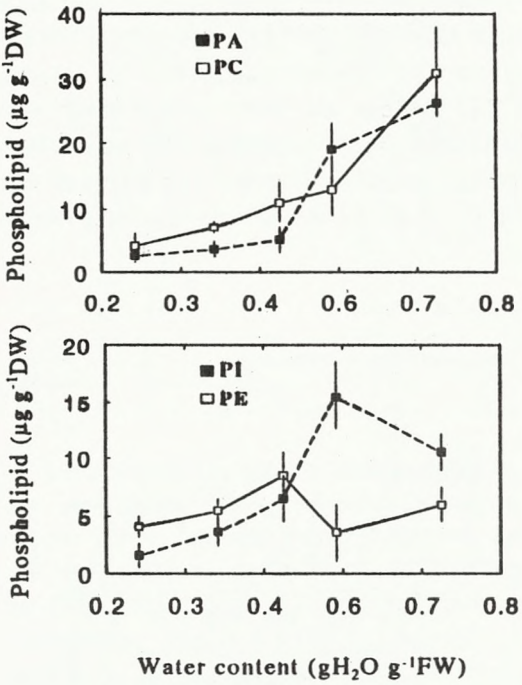


Fig. 3. Effect of low-temperature desiccation stress on the composition of phospholipids content: phosphatic acid (PA), phosphatidylcholine (PC); phosphatidylinositol (PI) and phosphatidylethanolamine (PE) in microsomal membranes of spruce primordial tissues. Results are ±SD from three independent measurements.

DISCUSSION

The effects of freezing and desiccation of spruce primordial shoots at −18°C leads to a loss of plasmalemma semipermeability, detected in this study as increased electrolyte leakage. These injury symptoms were apparent by 10th day of freezing and icreased in severity to day 40. Coincidently in the microsomal membrane fraction from primordial tissues phospholipids and proteins decrease (Fig. 2). Electron microscopic observations of freeze-fractured membrane preparations suggest that, following freeze stress, tissues are squeezed out of the gel-crystalline domains, resulting in protein-free areas within the membrane (Pearce and Willison 1985). Kendall et al. (1985) and Pukacki et al. (1991) also observed decrease of proteins and SH groups in the microsomal fraction of frozen crown tissue of wheat. They concluded that the decrease in membrane protein content occurred during the freezing and not during the thawing cycle. Our results demonstrate that the decrease of

membrane protein content of spruce shoot primordia tissue correlated with the loss of phospholipids. Proteins are generally lost from the membrane later and more slowly than phospholipids (Fig. 2). Hincha et al. (1987) suggested that *in vivo* dehydration both by freezing and desiccation, results in membrane rupture rather than in the dissociation of peripheral membrane proteins. A decreased amount of soluble proteins has also been reported for subfrozen winter rape leaves (Kacperska 1993).

Membrane phospholipid may also modulate the functions of membrane proteins. In the present investigation phospholipid classes are in general degraded during freeze-drought stress. Although the mechanism responsible for this type of phospholipid degradation is unknown, the action of lipolytic enzymes and/or attack by free radicals are possible causal agents (Senaratna et al. 1987). Phospholipase D is relatively well characterized and is known to be activated following freezing of woody tissues (Yoshida 1979). Following low-temperature stress the ability of membrane to maintain a semipermeable barrier was impaired and an increased leakage of cytoplasmic solutes was observed (Table 1). The nature of this lesion of membranes and the physical structure of the lipid bilayer of microsomal membranes from stressed and nonstressed tissues was investigated using microcalorymetry (Pukacki 1994). A lipid-phase transition temperature (T_m) of the membrane fraction from injured spruce tissues shifted from 10°C to 59°C (Pukacki 1994). It is known that PC tends to form a bilayer configuration, whereas PE forms an inverted hexagonal (H_{II}) configuration (Hoekstra and van Roekel 1988). It is now well established that freezing induced cellular dehydration results in the formation of lamellar-to-hexagonal and lateral phase transitions in the plasmalemma of plant cells (Webb and Steponkus 1993, Gordon-Kamm and Steponkus 1984). The loss of phospholipids and the reduction of thiol groups jointly indicate that frozen bud tissues have been exposed to oxidative stress during long-term freeze storage. There are two possible mechanisms by which phospholipid might be deesterified – enzymatically by phospholipase, or chemically by free radicals. It is unlikely that enzymes such as phospholipase are active in low temperatures (Sikorska and Kacperska 1982, Yoshida 1979).

In conclusion, degradation of membrane phospholipid during long-term freeze-drought stress of primordial shoots are interpreted as indicating enzyme catalyzed membrane deterioration. Hydrolysis of the ester bonds between the acyl groups and glycerol may be one of the main initial steps in phospholipid degradation, which may be mediated either by lipolytic enzymes or, alternatively, by free radicals possibly generated through perturbation of enzymes involved in electron transfer reactions.

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Właściwości błon komórek świerka pospolitego podczas długotrwałego stresu mrożenia

Streszczenie

Desykacja zawiązków pędów w pąkach świerka pospolitego (*Picea abies* (L.) Karst.) w temperaturze poniżej zera wywołuje w błonach mikrosomalnych szereg zmian fizycznych i biochemicznych. Pąki świerka poddane długotrwałemu (40-dniowemu) stresowi desykacji mrozowej w temperaturze −18°C wykazywały wyraźny wzrost przepuszczalności plazmalemmy dla jonów. Zawartość fosfolipidów (PL), białek oraz grup tiolowych (SH) izolowanych z mikrosomalnej frakcji błon ulega istotnej redukcji. Pierwsze wyraźne zmiany następują przy utracie przez tkanki 20% wody. Następuje wtedy spadek zawartości podstawowych fosfolipidów: fosfatydylocholiny, fosfatydyloinozytolu i kwasu fosfatydowego. Stwierdzono pozytywną korelację między zawartością fosfolipidów a spadkiem uwodnienia i stopniem uszkodzenia komórek związków. Stres desykacji mrozowej prowadzi również do degradacji białek i grup tiolowych błon.

Wyniki te sugerują że uszkodzenie komórek związków pędów świerka pospolitego poddanych długotrwałemu stresowi desykacji mrozowej jest następstwem strukturalnych i biochemicznych przemian w błonach cytoplazmatycznych.