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Justyna Nowakowska, Krzysztof Rakowski

Accelerated and natural ageing processes change the properties of plasma membrane in Norway spruce (*Picea abies* [L.] Karst.) seeds during storage

Abstract: The present study examines the mechanism of natural and accelerated ageing processes in Norway spruce (*Picea abies* [L.] Karst.) seeds stored at low temperature for one (control) and eight years. The analyses of vitality, electrolyte leakage, protein composition and activity of the proton-pump ATPase (P-ATPase, EC.3.6.1.35) in plasma membrane of Norway spruce seeds have been investigated. Seeds collected in 1999 from northeastern Poland have been treated with accelerated ageing method and compared to the seeds of the same provenance, collected in 1992 (control). The vigor and the vitality of the Norway spruce seeds depend on the age of seeds. After 6 days of experiment both samples of seeds (artificially aged seeds and control) expressed the same percentage of germination and similar electrolyte leakage. Analysis of protein composition in the extracts showed an increased amount of some low molecular-weight proteins in artificially and naturally aged seeds compared to the control. Our results indicate similar level of P-ATPase activity in natural, artificially aged and control seeds. This suggests that natural ageing process can involve changes in plasma membrane protein composition but does not affect the P-ATPase activity.

Additional key words: electrolyte leakage, P-ATPase, vigor, vitality

Address: Department of Genetics and Forest Tree Physiology, Forest Research Institute, Bitwy Warszawskiej 1920 street No 3, 00-973 Warsaw, Poland, e-mail: J.Nowakowska@ibles.waw.pl

Introduction

Norway spruce (*Picea abies* [L.] Karst.) is one of the most important forest tree species in Southern Poland. The conservation of the forest genetic resources in Poland mainly consists in the seed storage at low temperature and at the appropriate humidity.

The longevity of stored material depends on genetic factors and environmental conditions. The prolonged seed storage under low temperature conditions can cause several changes, which lead to fall of the viability percentage. It has been proved that during seed storage the electrolyte leakage *via* plasma membrane (Leinonen 1998) and the fluidity profile of the plasma membrane are increased (Rakowski *et al*. 1998). It is well known that the specific plasma membrane proton-pump P-ATPase enzyme (EC. 3.6.1.35.) is most important in active transport of molecules and ions. This way, it generates electrical potential, participates in signal transduction and it can be an important factor determining ageing process. In order to better understand the mechanism of natural and accelerated ageing process, we have compared vitality, electrolyte leakage, plasma membrane protein composition and activity of plasma membrane ATPase in Norway spruce seedlings grown from non-aged seeds (control), artificially aged (art. aged) and naturally aged (nat. aged) seeds.

Material and methods

Norway spruce (*Picea abies* [L.] Karst.) seeds (forest district Augustów provenance) collected in 1999 (stored for 1 year, further named as 1-year-old seeds)

and in 1992 (named as 8-year-old seeds) and were stored at – 3°C (moisture content 7.1%) in the dark.

The accelerated ageing was performed with 1-year-old seeds following K. Leinonen (1998) with modification at the time course of ageing (2–8 days).

According to method described by K. Leinonen (1998) we measured electrolyte leakage in different sets of seeds:

– non-aged seeds – control
– artificially aged seeds
– naturally aged (8-year-old) seeds.

Seeds were incubated for 24 hours in distilled water with shaking. Afterwards, electrolyte leakage was measured with conductometer (HANNA instruments Inc. apparatus HI 8733) at +22°C. Samples were homogenized and boiled at 100 °C for 10 sec. to determine 100% of membrane damage.

The vigor and the viability of seeds were evaluated by % of germination after 7 and 14 days (24°C, 39 $E \times m^{-2} \times s^{-1}$).

To measure the plasma membrane properties, the extracts were isolated from 10 g of frozen seeds according to Rakowski et al. (1998). The samples were stored at –72 °C in the dark.

The H^+-ATPase activity consisted in determination of inorganic phosphate (P_i), released from ATP (Lin and Morales 1977). The activity tests have been performed in the absence and in the presence of H^+-ATPase inhibitors: concanamycin (for vacuolar ATPase inhibition), sodium azide (mitochondrial ATPase inhibitor) and vanadate (plasma membrane ATPase inhibitor) as follow:

– buffer 5: 25 mM MES/Tris pH 6.5; 0,002% Brij 58; 0.1 mM sodium molybdate; 50 mM KCl,
– buffer X: buffer 5 supplemented by 5 nM concanamycin, 1 mM NaN_3,
– buffer 6: buffer 5 supplemented by 0.1 mM sodium vanadate.

The ATP hydrolysis has been determined after 30 minutes at 37°C incubation at the presence of 2.8 mM ATP and 3 mg of protein. The reaction was stopped by addition of the buffer containing 56.6 mM ammonium-hepta-molybdate; 2 mM ammonium-ortho-vanadate; 0.59 M nitric acid; 0.41 M NH_3; 2% sodium-dodecyl-sulfate (SDS).

The amount of released P_i in each sample was evaluated spectrophotometrically at 366 nm and calculated in mmol of P_i·mg protein $^{-1}$·h $^{-1}$. Protein level in particular fractions has been measured according to Bradford (1976).

To check the plasma membrane protein composition 14 mg of proteins extracted from 7-day-old seedlings has been separated by the SDS-PAGE electrophoresis system (Laemmli 1970) and stained with silver reagent (BioRad).

Results and discussion

The vigor and the vitality of the Norway spruce seeds depend on the age of seeds. The vigor and vitality of the accelerated aged and naturally aged seeds were lower than in the control and the percent of germination decreased during ageing treatment (Table 1). After 6 days of accelerated ageing treatment seeds express the same level of percent germination as the 8-year-old seeds. Moreover, the same treatment resulted in similar electrolyte leakage in natural and accelerated aged seeds compared to control (Fig. 1). Therefore, the membrane damage in artificially aged spruce seeds in the 6th day of treatment was comparable to damage caused by natural ageing process. For further analysis we have used the 6-day accelerated ageing treatment to compare natural and accelerated ageing processes.

The P-ATPase activity in the absence of inhibitors exhibits 20,3 mmol P_i·mg protein $^{-1}$·h $^{-1}$ in control; 18,4 mmol P_i·mg protein $^{-1}$·h $^{-1}$ in seedlings originated from accelerated aged seeds and 18.2 mmol P_i·mg protein $^{-1}$·h $^{-1}$ in seedlings originated from naturally aged seeds (Fig. 2). There was no significant ATP-hydrolysis inhibition in the presence of azide and concanamycin in the case of control and treatments. The use of vanadate in the reaction medium permitted to evaluate the relative degree of P-ATPase en-

Table 1. The percentage germination of the Norway spruce seeds submitted to accelerated ageing process, compared to the control (0 days of ageing) and naturally 8-year-old seeds. The percentage of germination in both cases: 6-day artificially aged seeds and naturally aged seeds is comparable (compare numbers in bold)

Accelerated ageing (days)	Germination 7 day-long (%)	Germination 14 day-long (%)
0	94	96
3.5	87	91
4.5	81	89
5	82	93
6	**62**	**78**
7	28	60
8	11	40
Naturally aged seeds (stored for 8 years)	**57**	**76**

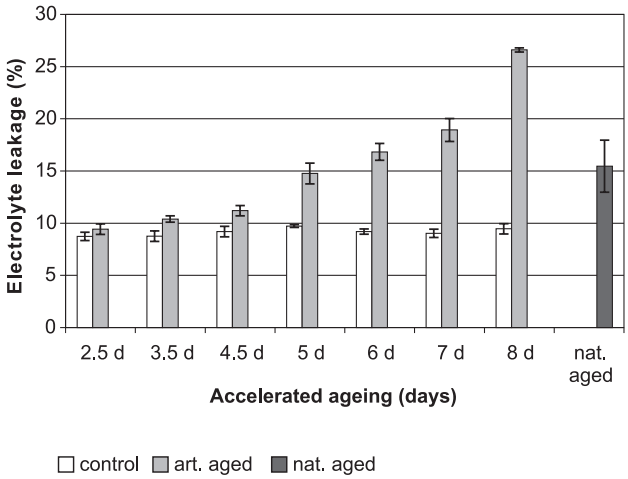

Fig. 1. Effect of accelerated ageing time on average electrolyte leakage in Norway spruce seeds from Augustów district forestry. Control: non-aged seeds collected in 1999. Art. aged: seeds collected in 1999 and submitted to accelerated ageing process. Nat. aged: naturally 8-year-old seeds from 1992. Bars represent means (n=3) ±SE

zyme purity in each sample. The presence of vanadate results in 50% inhibition of ATPase activity (Fig. 2). The 6-day accelerated ageing process caused an inhibitory effect on the P-ATPase activity similar to 8-year-old seeds (Fig. 2). Our results indicate similar level of P-ATPase activity in natural, accelerated aged and control seeds (Fig. 2). Preliminary analysis of

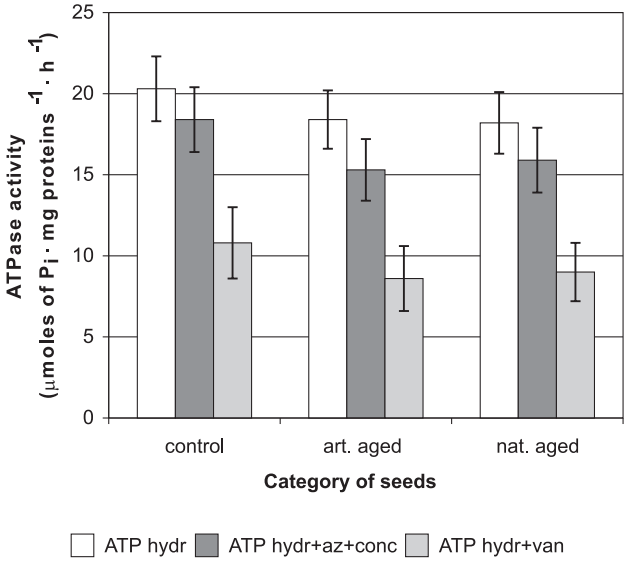

Fig. 2. Plasma membrane ATPase activity in Norway spruce 7-day-old seedlings (forest district Augustów). Control: seedlings germinated from non-treated seeds collected in 1999. Art. aged: seedlings from seeds collected in 1999 and submitted to accelerated ageing process. Nat. aged: seedlings germinated from seeds collected in 1992 (8-year-old). The ATP hydrolysis has been performed in the presence of inhibitors (azide, concanamycin and vanadate). Bars indicate means (n=4) ±SE

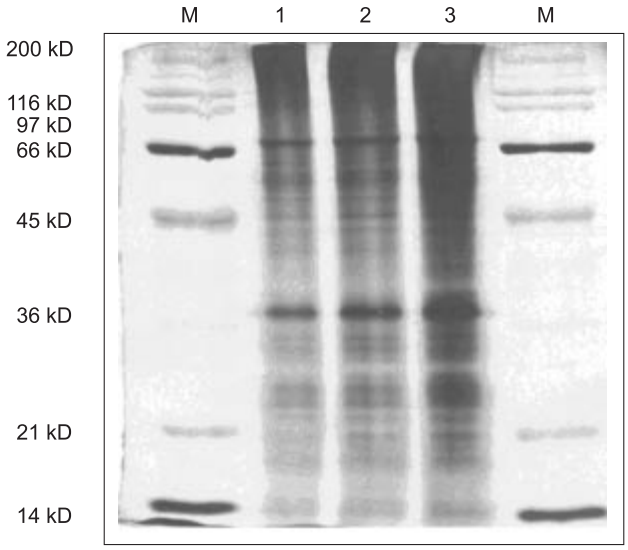

Fig. 3. Total protein separation of plasma membrane fraction in 14-day-old seedlings of Norway spruce on the SDS-polyacrylamide gel electrophoresis. Each lane contains 14 mg of proteins. M: molecular mass marker, lane 1: control, lane 2: protein extract from artificially aged seeds, lane 3: protein extract from 8-year-old seeds

protein composition in the extracts showed an increased amount of some protein bands separated by electrophoresis in accelerated and naturally aged seeds (Fig. 3). Those proteins (less than 40 kDa) may represent low molecular weight proteins of stress (like heat shock proteins – hsp or dehydrins, Leshem 1986; Kacperska 1998) or may result on an hydrolysis of proteins in plasma membrane during ageing.

Thus, the natural and accelerated ageing processes result in physiological changes involving decrease of seed vitality and increase of electrolyte leakage. In conclusion, natural ageing process can involve changes in plasma membrane protein composition but does not affect the P-ATPase activity.

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