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BRIEF COMMUNICATION

In vitro regeneration of mature *Pinus sylvestris* buds stored at freezing temperatures

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Abstract

Changes of morphogenic competence in mature *P. sylvestris* L. buds due to frozen storage were investigated. The highest callus formation was registered on explants stored at -18 °C for three months, but on explants stored for five months, it was also higher than in the control. Budding and development of needles *in vitro* was observed only for buds frozen three to five months. Peroxidase activity was lowest in these buds. In contrast, polyphenol oxidase activity in bud tissues continually increased during frozen storage. Within 10 months of frozen storage the content of starch and sugars in resting buds changed. It may be concluded that changes in composition of non-structural sugars in pine buds after five months of frozen storage are part of metabolic changes leading to loss of morphogenic capacity.

Additional key words: peroxidase, polyphenol oxidase, Scots pine, sugars.

Scots pine (*Pinus sylvestris* L.) is one of commercially most important conifer species in Northern Hemisphere. Tissue culture provides an important tool for practical propagation of selected genotypes of mature trees. However, no practically suitable method has been described so far for *P. sylvestris*.

Recent investigations have revealed that several peculiarities make mature Scots pine tissue culture difficult, *e.g.*, high oxidative stress during cultivation (Laukkanen *et al.* 2000), strong wound reactions accompanied by increase in phenolic substances (Hohtola 1988), and endophytic microbes (Pirttilä *et al.* 2002). As a result, rapid tissue browning, followed by deterioration of cellular ultrastructure and necrosis, as well as high percentage of infections usually occurs during tissue culture of *P. sylvestris*.

Our previous experiments with mature pine tissues revealed that morphogenic potential could be increased by cold storage of pine buds at 5 °C (Andersone and Ievinsh 2002). However, a relatively high percentage of infections during storage and a low rate of microshoots-forming explants were among major drawbacks of the method making effective propagation difficult.

A possibility to affect embryogenesis and morphogenesis by frozen storage of plant material was shown for other tree species (Bonga 1996). Yet, in spite of intensive practical use of cryopreservation of plant tissues for germplasm conservation (Sakai 2000, Mathur *et al.* 2003) there is practically no information available on biochemical changes during freezing storage of detached plant material.

The aim of the present work was to investigate the changes of morphogenic competence in mature *P. sylvestris* buds due to frozen storage. Peroxidase and polyphenol oxidase activities were measured as markers of oxidative metabolism. Starch and sugars content in buds was measured to investigate biochemical processes during storage as well as to elucidate the further nutritional demands of buds *in vitro*.

Terminal sections with buds and needles, about 15 cm long, were cut from branches in the lower half of the crown randomly from different mature pine (*Pinus sylvestris* L.) trees in a seed orchard near Riga, Latvia. Plant material was collected in winter of 2001 and 2002 (end of February with average daily temperature of -5 °C). The branches, together with a few handfuls of snow, were enclosed in plastic bags, which were quickly taken to a freezer running at -18 °C. Within 10 months of

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frozen storage branches were periodically in 1 to 4 months interval removed from the freezer. Buds were cut off from the branches and used as explants and for biochemical analyses. Each time three replicates of 20 buds per replicate were used as explants and three replicates were used for biochemical analysis.

To prepare the buds for tissue culture the following procedure was used. Buds were immersed in a solution of household soap for 1 h then rinsed in tap water for 1 h and surface sterilized with a partially diluted commercial bleach ACE (*Proctore and Gamble*, Riga, Latvia; containing 5 - 15 % sodium hypochlorite) for 20 min, rinsed again for 10 min in sterile distilled water, sterilized once more in 15 % H_2O_2 and rinsed three times for 10 min in sterile distilled water. Finally, the buds were peeled and dissected aseptically. It should be mentioned that only the upper part of buds was used as explants. For biochemical analyses peeled buds were used. For peroxidase and polyphenol oxidase measurements buds without scales (0.5 g) were frozen in liquid nitrogen and ground to fine powder with a mortar and pestle. For analyses of starch and sugars buds without scales (0.5 g for each replicate) were fixed in a hot steam for 20 min and dried.

Bud explants were cultivated in 20 × 200 mm glass test tubes containing 10 cm^3 nutrient medium solidified with agar. Tubes were closed with cotton-wool plugs and covered with thin polythene film fixed with a rubber band. Each tube contained one explant. They were cultivated at temperature of 23 ± 3 °C, 16-h photoperiod (irradiance of 40 - 45 μmol m^{-2} s^{-1}, fluorescent tubes *LB80-1* and *LB80-7* combined with sunlight).

Each time half of the explants were placed on medium for callus induction, and the other half were placed on medium for brachioblast and needle formation. For callus initiation explants were cultivated on a Murashige and Skoog medium modified by Hohtola (1988) with the exception that the only inorganic nitrogen source was 2 mM NH_4NO_3. As growth regulators, 4.5 μM 2,4-dichlorophenoxy acetic acid (2,4-D), 1.7 μM benzyladenine (BA), and 1.8 μM kinetin were used. A pH of the medium was adjusted to 5.6 - 5.7. For brachioblast and needle formation explants were cultivated on a Woody Plant Medium (WPM, Lloyd and McCown 1981) as modified by Andersone and Ievinsh (2002). 0.5 μM naphtyl acetic acid (NAA), 54 μM adenine, and 4.7 μM kinetin were used as growth regulators. The medium was supplemented with 0.6 mM myo-inositol, 88.9 μM thiamine hydrochloride, 48.6 μM pyridoxine hydrochloride, and 81.2 μM nicotinic acid. Explants were transferred to a fresh medium monthly.

To determine starch content, dried buds were ground with 10 cm^3 of solution containing 80 % (m/v) $Ca(NO_3)_2$ and boiled for 3 min to pass starch into the colloidal solution. The amount of starch was measured by Berthram method of bichromate-sodium thiosulfate titration (Strong and Koch 1974).

For measurement of non-polymeric sugars dried buds were ground to fine powder to which 10 cm^3 98 % (v/v) ethanol and 50 cm^3 of hot distilled water was added. Extraction was made in a water bath at 70 - 80 °C for 45 min. After extraction the material was supplemented with $Pb(CH_3COO)_2$ for sedimentation of proteins, fats and tannins. For the sedimentation of the remaining Pb ions 2 cm^3 of saturated Na_2SO_4 was added. The solution was filled up to 100 cm^3 with distilled water and filtered. The amount of reducing sugars in extracts was measured by Shaffer and Somogyi (1933) method of copper-iodometric titration (Strong and Koch 1974).

For determination of sucrose content, the total amount of sugars was measured first. Three cm^3 of 8 % (m/v) oxalic acid was added to 25 cm^3 of filtrate. To hydrolyze the sucrose, the solution was heated in boiling water for 10 min. After cooling a drop of methylene red was added and the solution was neutralized with 1 M NaOH until color became yellow. Distilled water was added up to 50 cm^3. The total amount of sugars was measured as described previously for reducing sugars. The content of sucrose was calculated by subtracting the content of reducing sugars from the total content of sugars and multiplying the result by 0.95. Sugar concentration was expressed as percentage of dry mass attributable to sucrose, starch, reducing sugars (hexoses glucose, and fructose), and total nonstructural sugars.

Enzymes were extracted from ground bud tissues with 25 mM HEPES/KOH buffer (pH 7.2) with addition of 1 mM EDTA, 3 % (m/v) insoluble polyvinylpyrrolidone, and 0.8 % (v/v) Triton X-100 for 15 min at 4 °C. The homogenate was centrifuged at 15 000 *g* for 20 min. Peroxidase and polyphenol oxidase activity was measured in the supernatant as described previously (Andersone and Ievinsh 2002).

Formation of callus on Hohtola's medium in control explants was observed within a month of cultivation (57 %, Table 1). The highest callus formation was registered on explants, stored for 3 months at -18 °C (100 %), but on explants, stored for 5 months, it was still higher (67 %) than in the control. Explants stored for six or more months did not form callus.

Budding and development of needles *in vitro* was observed only for buds frozen 3 to 5 months (20 - 30 % of explants on Woody Plant Medium, Table 1). Buds without freezing or those frozen for 6 or more months did not form any needles. Peroxidase activity was lower in buds, stored for 3 to 5 months, than in control (Table 1). Polyphenol oxidase activity in bud tissues continually increased during frozen storage (Table 1).

Within 10 months of frozen storage the content of starch and sugars in resting buds of mature *P. sylvestris* had changed. There were no statistically significant

Table 1. Changes of morphological characteristics *in vitro* and biochemical characteristics during frozen storage of mature resting pine buds. The ability to form callus was estimated for explants cultivated on Hohtola's medium. Needle formation was estimated on explants cultivated on Woody Plant Medium. Pine bud explants were taken from frozen material at the times indicated and placed on appropriate media. Morphological characteristics and sterility were evaluated a month later. Enzyme activities and sugar content were measured immediately after preparation of bud explants from frozen material. Equal letters for morphological parameters indicates non-significant differences at $P = 0.05$ between the time points using Student's *t*-test. Data are means from three replicates for every time point (± SE for biochemical measurements). For morphological characteristics 10 explants per replicate were measured. For sterility 20 explants per replicate were estimated.

	Time of storage [months]							
	0	1	2	3	4	5	6	10
Sterility [%]	43[a]	n.d.	n.d.	54[b]	n.d.	26[c]	10[d]	0
Callus-forming explants [%]	57[a]	n.d.	n.d.	100[b]	n.d.	67[c]	0	0
Needle-forming explants [%]	0	n.d.	n.d.	29[a]	n.d.	20[a]	0	0
Peroxidase activity [$\Delta A\ g^{-1}$(f.m.) min^{-1}]	95.6 ± 3.4	n.d.	n.d.	84.6 ± 2.6	n.d.	84.9 ± 3.3	98.4 ± 10.5	110.4 ± 5.8
Polyphenoloxidase activity [$\Delta A\ g^{-1}$ (f.m.) min^{-1}]	3.5 ± 0.9	n.d.	n.d.	6.5 ± 1.0	n.d.	8.7 ± 2.0	13.3 ± 0.2	19.2 ± 1.0
Sucrose [% d.m.]	7.50 ± 0.05	7.46 ± 0.10	7.64 ± 0.06	7.98 ± 0.09	6.76 ± 0.12	6.87 ± 0.03	6.13 ± 0.13	3.84 ± 0.14
Starch [% d.m.]	1.92 ± 0.05	1.61 ± 0.06	1.62 ± 0.03	1.36 ± 0.12	1.63 ± 0.05	1.58 ± 0.04	1.48 ± 0.05	1.38 ± 0.03
Glucose + fructose [% d.m.]	0.80 ± 0.05	0.63 ± 0.05	1.00 ± 0.02	1.21 ± 0.04	1.60 ± 0.06	1.65 ± 0.01	2.37 ± 0.07	4.16 ± 0.45

changes of the total content of non-structural sugars (data not shown).

However significant changes in the composition were found. The content of sucrose increased during first months of storage reaching maximum at 3 months followed by a decline (Table 1). In contrast, the content of reducing sugars (hexoses, glucose, and fructose) moderately increased up to 5 months with a sharp increase during the last part of the incubation period. The content of starch in pine buds slowly decreased during incubation. At the end of the experiment, the content of starch had decreased for about 31 %, the content of sucrose 58 %, but content of glucose and fructose together increased for 79 % (Table 1).

The results of the presented experiments with frozen storage of pine buds before introducing them *in vitro* suggest that such storage of detached material could be used for affecting metabolic processes in Scots pine tissues. Only limited information is available on biochemical changes during prolonged storage of frozen plant material. In contrast to widespread opinion that frozen state represents a situation of arrested metabolism, the present experiments clearly showed that during frozen storage of pine buds at -18 °C significant changes of sugar metabolism and oxidative enzyme activities occur.

Due to frozen storage, morphogenic capacity considerably increased in pine buds stored for 3 months and, to a lesser extent in buds stored for 5 months, in comparison with control buds introduced in a culture immediately after collecting. It was revealed by our previous experiments (Andersone and Ievinsh 2002) that lowered activity of oxidative enzymes in pine tissues might contribute to better morphogenic potential. In the present experiments, increased ability to form callus and needles coincided with the lowest peroxidase activity in frozen stored pine buds (Table 1). In addition, the period of highest morphogenic capacity coincided with a highest sucrose content.

In leaf buds of pines, extracellular freezing occurs in response to subfreezing temperatures, in contrast to extra-organ freezing in leaf buds of other conifers (Ide *et al*. 1998). This leads to extremely high freezing tolerance of pine bud tissues. In our experiments, Scots pine buds were collected in winter in a state of full adaptation to low temperature conditions. Therefore, biochemical changes in plant tissues related to cold acclimation should be taken into account for understanding the results of the present experiments. Increase of soluble sugars, mostly sucrose, usually occurs in plants during cold hardening in parallel with decline in the content of insoluble sugars (Oleksyn *et al*. 2000). It is thought that increase in sugar concentration is a result of the degradation of starch (Fischer and Höll 1991). Accumulation of soluble sugars can prevent membrane injuries during freezing (Fujikawa and Jitsuyama 2000). In addition, during low-temperature storage free radical mediated oxidative stress occurs (Hendry 1993).

Therefore, increase in antioxidant enzyme activities is essential for cold acclimation (Tao *et al*. 1998). However, increased oxidative activity in mature pine tissues is among factors leading to decreased morphogenic potential (Andersone and Ievinsh 2202).

It may be concluded that changes in composition of non-structural sugars in pine buds after 5 months of frozen storage are part of metabolic changes leading to loss of morphogenic capacity.

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