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Growth of tissue culture and changes in oxidative enzyme activity of *Sorbus* and tayberry cultivars during cold storage

Dace Kļaviņa[1]*, Gederts Ievinsh[2]

[1]Tissue Culture Department, National Botanical Garden, Miera 1, Salaspils LV-2169 Latvia
[2]Department of Plant Physiology, Faculty of Biology, University of Latvia, Kronvalda Bulv. 4, Rīga LV-1586, Latvia

*Corresponding author, E-mail: dace.klavina@nbd.gov.lv

Abstract

The aim of the present work was to compare the effect of different concentrations of sucrose on success of long-term cold storage of two woody plant tissue cultures – *Sorbus* and tayberry cultivars. Increased sucrose concentration significantly diminished the number of necrotic shoots for *Sorbus* explants. High sucrose concentration in the medium together with other optimal cultivation conditions allowed successful storage of *Sorbus* and tayberry cultivars without subculturing for five years. After transplanting to fresh medium at 24 °C explants completely renewed growth and development. During the period of initial growth inhibition at low temperature the activity of oxidative enzymes peroxidase and polyphenol oxidase increased, reaching a peak value when growth of the explants resumed. Increase of both peroxidase and polyphenol oxidase activity during the period of growth inhibition was positively correlated with the concentration of sucrose in the incubation medium.

Key words: cold storage, oxidative enzymes, *Sorbus*, sucrose, tayberry, tissue culture.

Introduction

Cold storage or slow growth of plant tissue cultures is widely used as a method for maintenance of *in vitro* collections of plant tissues (Blakesley et al. 1996). The cold storage success of each taxon is determined genetically (Engelmann et al. 1997; Palonen, Buszard 1998), but it also depends on storage temperature, illumination, cultivation medium composition as well as preconditioning of tissue cultures before low temperature application (Wilson et al. 1998; Jouve et al. 2000). Addition of sucrose to the medium increases dry mass and maintains overall plantlet quality during cold storage (Wilson et al. 1998; Palonen, Junttila 1999).

Transfer to cold induces expression of wide array of genes, many of which are related to adaptation to cold (Chinnusamy et al. 2006). From the first days of low temperature storage explants undergo significant biochemical change in oxidative metabolism (Jouve et al. 2000). Peroxidases and polyphenol oxidase are important constituents of enzymatic oxidative metabolism, leading to formation of bioactive phenolic compounds (Mika et al. 2004; Mayer 2006). Consequently, increased activity of peroxidase or polyphenol

oxidase in cultivated plant tissues during cold storage may be an indication of deleterious metabolic changes.

The aim of the present experiments was to compare the effect of different concentrations of sucrose on success of long-term cold storage of two different commercially important woody plant tissue cultures – *Sorbus* and tayberry cultivars. Oxidative enzyme activity was monitored as an indicator of oxidative metabolism.

Materials and methods

The *Sorbus* cultivar ‚Krasnaja Krupnaja' and tayberry cultivar ‚Medana' maintained for several years in tissue culture were used for cold storage experiments. As explants, shoot fragments (2 cm in length) from proliferating culture were planted on Murashige-Skoog medium with addition of 0.3 mg L^{-1} 6-benzylaminopurine) and different concentrations of sucrose (30, 40, 60 g L^{-1}). The cultures were adapted on the medium at 25 °C (photoperiod 16 h, PAR 40 μmol s^{-1} m^{-2}) for three weeks before transfer to low temperature (5 °C, photoperiod 16 h, PAR 20 μmol s^{-1} m^{-2}) cold storage.

Samples for enzyme analysis during the first month of cold storage were taken every two weeks, later – once a month. Enzymes were extracted from frozen explant tissues ground to fine powder with 25 mmol L^{-1} HEPES/KOH buffer (pH 7.2) containing 3 % polyvinylpolypirrolidone (w/v). Peroxidase (EC 1.11.1.7) and polyphenol oxidase (EC 1.10.3.2) activity was measured spectrophotometrically as described previously with guaiacol plus H_2O_2 and pyrocatechol as substrates, respectively (Andersone, Ievinsh 2002). Four replicates of enzyme determination for each time point were performed.

Results

Sorbus explants were successfully cultivated for 5 years in conditions of cold storage at 5 °C. Three media differing in sucrose concentration were used in the present experiments

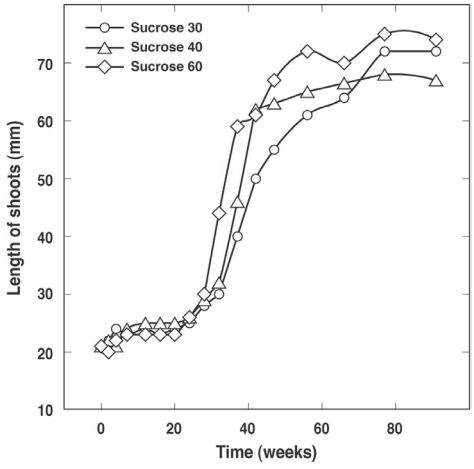

Fig. 1. Growth of shoot explants of *Sorbus* cv. ‚Krasnaja Krupnaja' at different sucrose concentrations during cold storage at 5 °C.

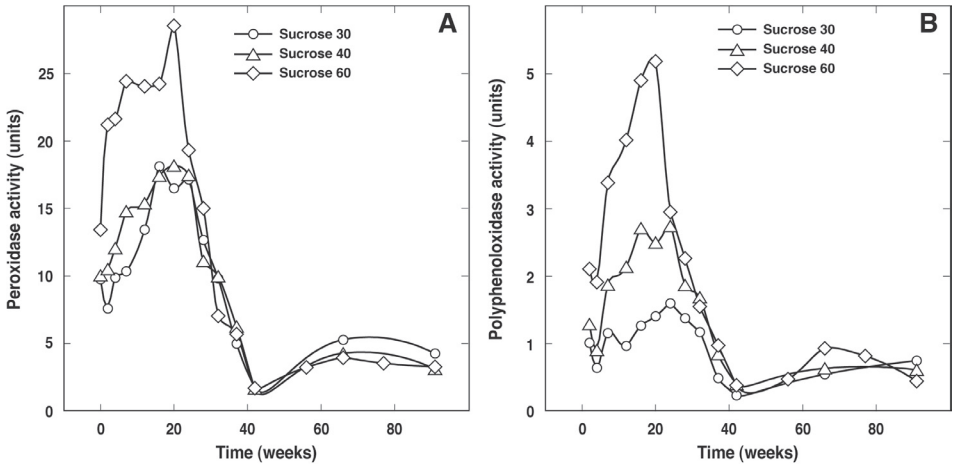

Fig. 2. Time course of peroxidase (A) and polyphenol oxidase (B) activity in explants of *Sorbus* cv. ‚Krasnaja Krupnaja' at different sucrose concentrations during cold storage at 5 °C.

for cold storage. Complete growth inhibition of *Sorbus* explants was evident at all sucrose concentrations used for cultivation from week 10 till 20 (Fig. 1). During that period activity of oxidative enzymes peroxidase and polyphenol oxidase increased reaching a peak value at the time when growth of the explants resumed (Fig. 2). The growth inhibition-related increase in oxidative enzyme activity depended on the sucrose concentration used for cultivation – the increase was highest at 60 g L^{-1} sucrose concentration. However sucrose concentration did not affect growth characteristics of *Sorbus* explants in the growth inhibition stage during cold storage. When intensive growth of explants started after 37 weeks of cultivation in cold conditions, explants grown at higher sucrose concentration (40 and 60 g L^{-1}) had a tendency to have higher growth rates (Fig. 1). However the effect was not seen at later stages. Cytokinin stimulated formation of adventitious shoots (1.3 to 1.5 per initial explant in average). Adventitious shoot formation was not affected by the sucrose concentration used. However after 91 weeks of cold cultivation, an increased concentration of sucrose in the medium diminished the amount of necrotic shoots (Table 1). The effect was more pronounced for the main shoots in comparison to adventitious shoots. At the highest sucrose concentration (60 g L^{-1}) all explants were viable after 5 years of cold storage without subcultivation. No root formation was evident on *Sorbus* explants

Table 1. Effect of sucrose concentration on the number of necrotic shoots (as % from initial explants for main shoots or % from newly formed adventitious shoots) on *Sorbus* explants after 91 weeks of cultivation at 5 °C

	Number of necrotic shoots (%)	
Sucrose concentration (g L^{-1})	**Main shoots**	**Adventitious shoots**
30	55 ± 5	32 ± 4
40	30 ± 4	28 ± 2
60	15 ± 2	17 ± 3

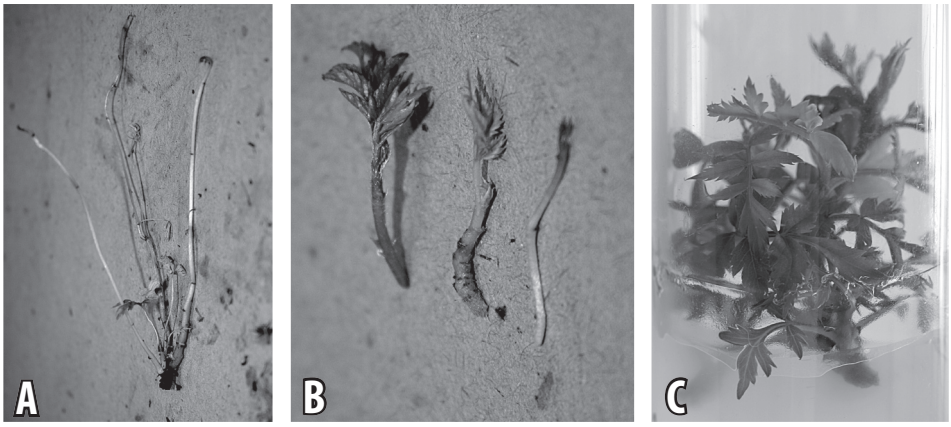

Fig. 3. A, microshoots formed from one explant of *Sorbus* cv. ‚Krasnaja Krupnaja' after 5 years of cold storage at 5 °C. B, cold-stored explants before transfer to a fresh medium. C, the same explants after one month at 24 °C.

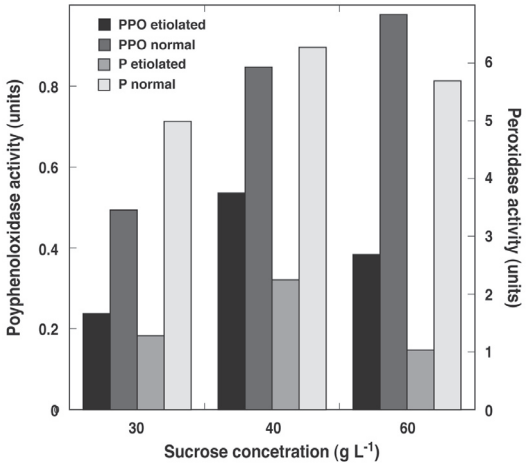

Fig. 4. Effect of morphological characteristics of shoots formed on *Sorbus* explants stored at 5 °C for 37 weeks on peroxidase and polyphenol oxidase activity during the stage of active growth.

during cultivation. In contrast, at lower sucrose concentrations all explants died after 3 to 4.5 years of cold cultivation.

Sorbus microshoots from explants cultivated 5 years in the cold were suitable for further propagation (Fig. 3A, B). After explanting to fresh medium at 24 °C they completely renewed growth and development (Fig. 3C).

After transfer from 24 °C to cold storage conditions (5 °C) the activity of both oxidative enzymes examined increased in cultivated tissues of *Sorbus*, reaching maximum values 16 to 24 weeks after the start of cold storage (Fig. 2). The increase was most pronounced at highest sucrose concentration (60 g L^{-1}), being five times and three times that of initial activity, for polyphenol oxidase and peroxidase, respectively. However the difference disappeared during the latter stages of cultivation. During activated shoot growth there

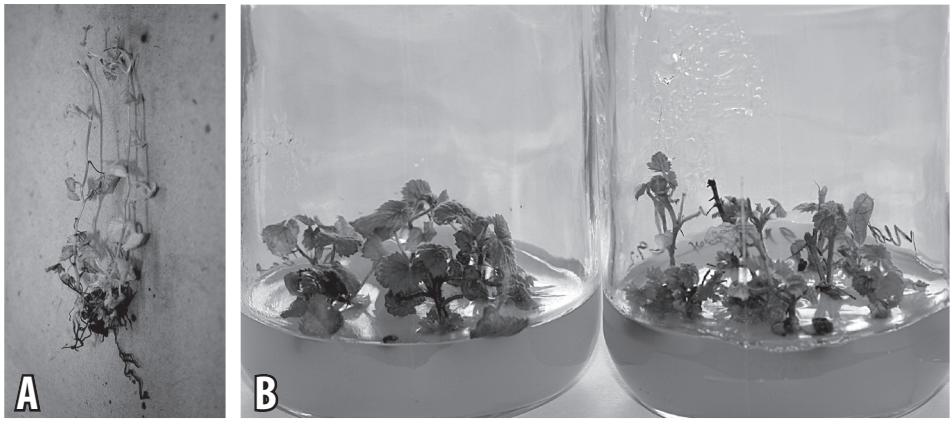

Fig. 5. A, microshoots formed from one explant of tayberry ‚Medana' stored at 5 °C for 5 years (sucrose 30 g L^{-1}). B, regrowth of new explants after transfer to a fresh medium containing sucrose 40 g L^{-1} (left) or 60 g L^{-1} (right).

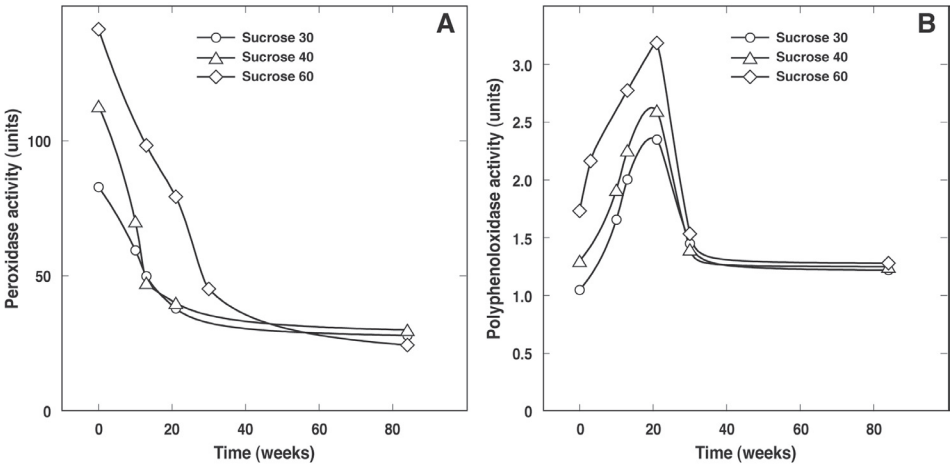

Fig. 6. Time course of peroxidase (A) and polyphenol oxidase (B) activity in explants of tayberry ‚Medana' at different sucrose concentrations during cold storage at 5 °C.

was a dramatic decrease of enzyme activities.

At the stage of growth activation from week 30 oxidative enzyme activity was analyzed separately in normal and extremely elongated etiolated shoots. Etiolated shoots had an average fresh mass of 29 ± 5 mg in contrast to 67 ± 7 for normal shoots. Elongated shoots had significantly lower activity of both polyphenol oxidase and peroxidase at all tested sucrose concentrations (Fig. 4).

Tayberry cv. ‚Medana' explants were successfully cultivated at 5 °C for 5 years. Growth of explants was less inhibited than in the case of *Sorbus* explants. In addition, in contrast to *Sorbus* where only the highest sucrose concentration (60 g L^{-1}) in the medium was suitable for long storage, tayberry explants cultivated at all sucrose concentrations (30, 40, 60 g L^{-1}) were completely viable and suitable for further micropropagation (Fig. 5A). After transfer

to a fresh medium these explants developed normally at 24 °C (Fig. 5B).

Root formation was monitored during cold storage of tayberry explants at all sucrose concentrations, during the period of low peroxidase activity (21 to 30 weeks of cultivation; Fig. 6).

Tayberry cv. ‚Medana' explants showed a similar time course of oxidative enzyme activity during cold storage as found for *Sorbus* explants (Fig. 6). However the period of cold transfer-induced increase in the activity was relatively shorter, especially for peroxidase activity. Increase of both peroxidase and polyphenol oxidase activity during the period of growth inhibition was positively correlated with the concentration of sucrose in the medium. Average peroxidase activity in tayberry explants was about 10 times that in *Sorbus* explants, while the average polyphenol oxidase activity in both cultures was in the same range.

Discussion

Successful storage without subculturing for woody plant species usually is up to two years. The longest cold storage reported so far is 60 months for *Eucalyptus grandis* shoot explants (Hausman et al. 1994). In the present work storage without subculturing of equivalent length was achieved by *Sorbus* and tayberry cultivars. The explants were suitable for further micropropagation with vigorous regrowth at 24 °C.

Growth inhibition is a necessary prerequisite for preservation of plant tissues by a slow growth technique. Thus, cold incubation-induced slow growth of plant tissue cultures resembles natural dormancy period of vegetative tissues in the temperate region. However, three phases in respect to growth processes were clearly distinguishable during cold storage of both *Sorbus* and tayberry explants in the present experiments: growth inhibition for a initial 20 weeks of cold incubation followed by a relatively rapid shoot elongation (30 to 40 weeks), and further a reduced but relatively stable growth rate. Similar to our observations, it was already described earlier that woody plant cultures grow during cold storage (Pruski et al. 2000).

During the first phase of cold incubation explant tissues undergo cold acclimation. Low temperature as a signal acts through abscisic acid-dependent signaling pathways leading to physiological and biochemical changes characteristic for „end of summer" responses (Welling, Palva 2006). Increased protection against endogenous oxidative stress is one of the prerequisites of cold acclimation (Renaut et al. 2005). Thus, growth inhibition and an increase in peroxidase activity in the present experiments is related at the level of both nonspecific antioxidative protection as well as to general oxidative metabolism together with polyphenol oxidase. A distinct relationship between a relatively high activity of oxidative enzymes and a minimum growth rate was evident during the first phase of cold storage. As sucrose stimulated both peroxidase and polyphenol oxidase activity a casual relationship between cold-induced suppression of growth and increased oxidative metabolism can be suggested. The low level of activity of both enzymes during the second and the third phase of cold storage clearly indicated the absence of any oxidative-stress related metabolic disorders for all experimental treatments in spite of shoot necrosis at lower sucrose concentration for *Sorbus* explants.

Sucrose concentration in the medium was extremely important for successful cold storage of *Sorbus* explants. An increased sucrose concentration significantly diminished

the number of necrotic shoots (Table 1), and 100 % viability after five years of cold storage was achieved. In addition, there was a positive correlation between sucrose concentration and oxidative enzyme activity during the period of growth inhibition of both *Sorbus* and tayberry explants. Significant differences in oxidative enzyme activity as noted for *Sorbus* and tayberry explants was described previously also for *Rubus* and *Cerasus* cultivars (Klavina et al. 2001).

The presence of sucrose in the medium has been shown to be of critical importance during prolonged cold storage of several plant cultures (Pruski et al. 2000). A high sucrose level during *in vitro* cultivation of olive microshoots was important for further cold tolerance (Bartolozzi et al. 2001). In cold acclimation, during the initial stage of slow growth sucrose might act both as an osmoprotectant (Nagao et al. 2005; Suzuki et al. 2006) as well as one of the signals for successful growth inhibition of explants (Rolland et al. 2002). It is well known that many stress- and abscisic acid-inducible genes are coregulated by sugars (Rolland et al. 2002).

In conclusion the present experiments clearly showed that for woody plant cultivars e.a. *Sorbus* and tayberry high sucrose concentration is of extreme importance during cold incubation-induced slow growth storage. Together with other optimal cultivation conditions, storage without subculturing for five years is successful.

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Pīlādžu un kazenes-avenes hibrīda augšana audu kultūrā un oksidatīvo fermentu aktivitātes izmaiņas aukstuma uzglabāšanas laikā

Dace Kļaviņa[1]*, Ģederts Ieviņš[2]

[1]Audu kultūru nodaļa, Nacionālais botāniskais dārzs, Miera 1, Salaspils LV-2169, Latvija
[2]Augu fizioloģijas katedra, Latvijas Universitātes Bioloģijas fakultāte, Kronvalda bulv. 4, Rīga LV-1586, Latvija
*Korespondējošais autors, E-pasts: dace.klavina@nbd.gov.lv

Kopsavilkums

Šī darba mērķis bija salīdzināt dažādu saharozes koncentrāciju ietekmi uz divu kokaugu kultūru – pīlādžu un kazenes-avenes hibrīda – ilgstošu uzglabāšanu pazeminātā temperatūrā. Paaugstināta saharozes koncentrācija ievērojami samazināja nekrotisko dzinumu daudzumu pīlādžu eksplantiem. Augsta saharozes koncentrācija barotnē kopā ar optimāliem kultivēšanas apstākļiem ļāva veiksmīgi uzglabāt pīlādžu un kazenes-avenes hibrīdu šķirnes piecus gadus bez pārstādīšanas. Pēc pārstādīšanas svaigā barotnē eksplanti pilnībā atjaunoja augšanu un attīstību 24 °C. Sākotnējās augšanas inhibēšanas laikā pazeminātā temperatūrā oksidatīvo fermentu peroksidāzes un polifenoloksidāzes aktivitāte paaugstinājās, sasniedzot maksimumu brīdī, kad eksplantu augšana atjaunojās. Abu fermentu aktivitātes pieaugums augšanas inhibēšanas laikā pozitīvi korelēja ar saharozes koncentrāciju barotnē.