# *IN VITRO* CULTURES AND CRYOPRESERVATION AS A TOOL FOR CONSERVING OF FRUIT SPECIES

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#### ABSTRACT

Apple (Malus domestica Borkh.), pear (Pyrus communis L.), sour cherry (Prunus cerasus L.) and strawberry (Fragaria ananassa Duch.) are important fruit species in the Czech Republic. These species are susceptible to a number of diseases, which could damage field-grown materials. Cryopreservation of in vitro-grown shoot tips is a suitable option for the safe, long-term storage of germplasm of vegetatively propagated fruit species. Fifty genotypes of apple, pear, sour cherry and strawberry were successfully established in vitro and were micropropagated on Murashige and Skoog (MS) based media. The highest proliferation rate was obtained for pear cultivar 'Koporecka' that produced 5.2 new shoots per month on MS medium supplemented with 1 mg 1-1 BAP (6-benzylaminopurine). Survival of in vitro propagated pear, apple and sour cherry shoots and strawberries was evaluated after low temperature treatment. Cold hardening and preconditioning improved the recovery of cryopreserved shoot tips. A procedure based on the encapsulation-vitrification of samples has yielded particularly promising results, with a survival rate of 30% or more across a number of genotypes. Apple, pear, sour cherry and strawberry germplasm (50 genotypes) is now stored in the cryopreserved form in liquid nitrogen at −196°C.

#### INTRODUCTION

Cryopreservation techniques (including cryopreservation of in vitro grown shoot tips) that are effective for a wide range of genotypes, are important for both genebanks and breeders collections. These techniques save time and labor costs and increase the security of germplasm collections (Chang and Reed 2001). This work presents the methods for long-term storage of 50 genotypes of apple, pear, sour cherry and strawberry.

### MATERIALS AND METHODS

In vitro cultures of apple, pear, sour cherry and strawberry were established from field grown plants in genebank of RBIP Holovousy Ltd. in years 2001–2004. Shoot tips of apple, pear and sour cherry (0.5-1 cm in length) were cut after bud break. Apical meristems (size 1-2 mm) were prepared from runners of strawberries under a stereoscopic microscope. This initial plant material was surface disinfested with a 0.15% solution of mercuric chloride for 1 min. The explants were each placed into Erlenmayer flasks (100 ml) with shaped narrow neck and metal caps. Culture media were based on the mineral salts and organic constituents of Murashige and Skoog (1962) supplemented with different concentrations of growth regulators: 0.5 or 1 mg l<sup>-1</sup> TDZ (thidiazuron), 1, 2 or 4 mg l<sup>-1</sup> BAP (6-benzylaminopurine) or 1 mg l<sup>-1</sup> BAP in combination with 1 mg l<sup>-1</sup> IBA (indolebutyric acid). Auxins and cytokinins were filter sterilised (25 mm Acrodisc Syringe Filter 0.2 µm, Pall Gelman, US) and added after autoclaving. Cultures were incubated under 16-h photoperiod provided by cool-white fluorescent tubular lamps at 60 µmol m<sup>-2</sup> s<sup>-1</sup> and 23±1°C.

Established in vitro cultures were cold hardened for 4 to 6 weeks in a cold hardening chamber at 4°C, under a 16 h light/8 h dark photoperiod and light intensity 25  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>. Shoot tips from cold hardened in vitro cultures were excised, soaked with 2M sucrose for 48h, encapsulated in 3% alginic acid in 0.75M sucrose and then dropped into the 0.1M CaCl<sub>2</sub> in 0.6M sucrose to net the alginate. The beads were placed in a laminar flow box at laboratory temperature, dehydrated for 4 to 6 h (to the level of 35% fresh weight) and plunged in liquid nitrogen. After at least 1 week, the encapsulated shoot tips were thawed by plunging in 40°C water and placed on MS medium in cultivation chamber at 23°C, long photoperiod (16/8, light/dark) and light intensity 109 µmol m<sup>-2</sup> s<sup>-1</sup>. The survival and regrowth were evaluated after 14 and 28 days at regeneration conditions. Survival was defined as the percentage of the total number of shoot tips that showed green color 14 days after thawing and culture. Shoot regrowth was recorded as the percentage of the total number of shoot tips forming at least one new shoot 28 days after thawing and culture.

In all experiments 20 to 30 shoot tips were used. Each experiment was repeated three times. Data from three independent experiments were pooled and expressed as the mean.

## **RESULTS AND DISCUSSION**

Four species (apple, pear, sour cherry and strawberry) and 50 genotypes were tested in this study. Sterilization procedures were successful and less then 10% of initial explants of any genotype became visibly contaminated with microorganism. Shoot number and shoot morphology varied with the genotype and with the concentration of different plant hormones. The highest multiplication rate for the pear cultivar 'Koporecka' was obtained on the medium with BAP concentration 1 mg l-1, which produced 5.2 new shoots per explant. The concentration of BAP 1 mg l-1 was broadly efficient and applicable for multiplication of apple, pear and sweet cherry genotypes. Concentration of BAP  $(2 \text{ mg } l^{-1})$  resulted in production of mostly short (0–0.5 cm) shoots with abnormally narrow leaves, which were difficult to count. Further increase in BAP concentration had unfavourable effect on shoot number and shoot morphology of many cultivars. Explants on media with 4 mg l<sup>-1</sup> BAP frequently turned brown and deteriorated. The combination of 1 mg l<sup>-1</sup> BAP with 1 mg l<sup>-1</sup> IBA was the most favourable for multiplication of strawberry genotypes. All the tested strawberry genotypes had the multiplication coefficient higher than

3.5 on this medium. TDZ induced many small callused shoots with high level of hyperhydricity, which did not elongate and were difficult to count, particularly at a concentration of 1 mg  $l^{-1}$ .

The response of fruit crops on the encapsulation-dehydration cryopreservation protocol was different. The mean survival and regrowth of cryopreserved shoot tips of 10 apple cultivars were 55% and 43% respectively. Apple cultivar 'McIntosh' had the highest (85%) and 'Zvonkove' had the lowest regrowth (4%). The mean survival and regrowth of cryopreserved shoot tips of 6 pear cultivars were 41% and 33% respectively. Pear cultivar 'Koporecka' had the highest (67%) and 'Clapova' had the lowest regrowth (14%). The mean survival and regrowth of cryopreserved shoot tips of 5 sour cherry cultivars were 20% and 14% respectively. Prunus x ceropadus had the highest regrowth (42%). The mean survival and regrowth of cryopreserved shoot tips of 4 strawberry cultivars were 22% and 15% respectively. Strawberry cultivar 'Lidka' had the highest regrowth (30%). 'Senga Sengana' formed callus.

All the shoots developed from cryopreserved shoot tips had the same morphological appearance as the initial in vitro cultures. New shoots were produced by direct development of apical dome.

The results obtained in our work the confirmed previous studies on *Pyrus communis* genotypes (Sedlak and Paprstein 1999), which claimed that increasing BAP concentrations significantly reduced the number of shoots with normal leaves. An ideal proliferation medium should optimise not only the number of shoots produced, but also their quality. In our study TDZ induced many small shoots, which did not elongate and were difficult to count. Similar observations on the effect of TDZ were recorded in in vitro cultures of apple *Malus* sp. (Sarwar et al. 1998).

Wu et al. (2001) studied the effect of cryopreservation methods on different apple cultivars. They found the encapsulation dehydration method the most effective in comparison to other types of cryopreservation methods (twostep freezing and vitrification). They reached similar rates of regeneration after cryopreservation procedure (86% for apple cultivar 'Jonagold') as was obtained in our results. On the other hand, they did not obtain such low survival and regeneration rate as we did in cultivar 'Zvonkove'. Sensitivity of 'Zvonkove' cultivar to cold temperature treatment could be the possible reason for such a low regeneration (Bilavcik 2003).

The survival of encapsulated shoot tips of in vitro cultures strongly depends on the water content in their tissues. Scottez et al. (1992) found that dehydration up to the 20% of residual water was optimal for pear cv. 'Beurre Hardy' encapsulated in alginate beads. They obtained the highest regeneration up to 80%. In our work only cv. 'Koporecka' reached comparably high level of regeneration (67%). Regeneration of other cultivars ranged from 14% to 43%. The results from encapsulation dehydration cryopreservation applied on strawberry and cherry cultivars showed big fluctuation of survival and regeneration rates among different cultivars. It suggests that it is necessary to adapt the cryopreservation method to the individual demands of species or cultivars according to the thermal characteristics and dehydration tolerance of their shoot tips.

Duplicate collections (50 cultivars, 4 species) of fruit crops are now stored in the Cryobanks of RICP Praha Ruzyne and RBIP Holovousy Ltd.

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