

# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

185,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index  
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?  
Contact [book.department@intechopen.com](mailto:book.department@intechopen.com)

Numbers displayed above are based on latest data collected.  
For more information visit [www.intechopen.com](http://www.intechopen.com)



# Comparison of Cryopreservation Methods of Vegetatively Propagated Crops Based on Thermal Analysis

Jiří Zámečník, Miloš Faltus, Alois Bilavčík  
and Renata Kotková  
Crop Research Institute  
The Czech Republic

## 1. Introduction

There is a trend to preserve the plant germplasm by not only conventional *ex situ* methods or *in vitro* techniques, but also, more recently, by cryopreservation. Cryopreservation techniques are based on the storage of plant samples at very low temperature at which practically no chemical reactions occur and consequently, neither aging nor genetic changes of plant material. There has been a great development progress of cryopreservation methods during last years. Cryopreservation becomes a highly utilized technique for germplasm conservation. Generally the cryopreservation is storage of the samples. The samples can be e.g. organs and shoots tips from *in vitro* culture, or from the field, such as mature, immature bulbils, cloves of garlic or dormant buds of fruit trees, at the ultra-low temperature (mainly  $-196^{\circ}\text{C}$ , the temperature of liquid nitrogen).

Although the technique was introduced for plants in the '70s, it has never been applied on a wide scale due to the high cost of cryo-freezers; indeed, it was used in order to escape the formation of lethal intracellular ice crystals, time-consuming and laborious slow-cooling procedures. A new cryogenic - vitrification technique is now available, aiming at the direct immersion of plant specimens from tissue cultures in liquid nitrogen, without resorting to an expensive apparatus for slow cooling and with a considerable simplification of the procedures (Benson, 2008). The vitrification method simplifies cryogenic process and makes possible an increased application of cryopreservation on wide-range plant genetic resources. The glassy state is the objective status of cryopreservation methods named vitrification.

The aim of this study is a comparison of different cryopreservation methods based on the vitrification achieved by dehydration and glass transition temperature ( $T_g$ ), and their efficiency towards optimal regeneration of vegetatively propagated plants. The thermal characteristics, evaluation of frozen water content, and the glass transition temperature were measured by a differential scanning calorimeter.

## 2. Importance of cryopreservation of vegetatively propagated plants

Some of vegetatively propagated plants are not able to reproduce by seeds e.g. garlic plant (*Allium sativum* L.). The only way how to propagate it is to use its cloves or bulbils

for seeding plants for further growing. The vegetatively propagated plant germplasm is endangered by abiotic and biotic factors in the field conditions. Although the production area of many vegetatively propagated plants has been decreasing, many local cultivars and varieties remain. In the presence of decreasing cultivar variability in production areas, diminishing of old orchards, as well as appearance of diseases close to field collection areas, the question of safely maintaining the broad genetic potential of fruit trees is arising.

Two safe methods ensure vegetatively propagated plant germplasm maintenance with a low risk of loss: slow-growth *in vitro* culture and the cryopreservation methods. Advantages of *in vitro* collection are aseptic and stable conditions of the cultivation and availability of the material during the year. A disadvantage is the necessity of sequential plant multiplication. Advantages of cryo-collection are low costs for its long-term maintenance and material stability. Disadvantages are a longer time for the plant to recover from stored material and a rather high input costs of the cryopreservation procedure. The best way how to maintain germplasm is the combination of both methods. The base collection should be maintained by *in vitro* collection that provides the material in case of requirements. Core collection of the most valuable material, should be backed-up by cryo-collection for long-term storage, and plants are recovered just in case the genotype is lost from the base collection. For that reason, important vegetatively propagated plant collections have started to introduce accessions to slow-growth *in vitro* cultures and simultaneously in cryo-collection in liquid nitrogen (Gonzalez-Arno *et al.*, 2008; Keller *et al.*, 2008; Kim *et al.*, 2006).

### 3. Cryopreservation methods

The latest results from the field of low temperature biology suggest that the main factor influencing the success of the cryopreservation method is the maintenance of a glassy state in plant samples and the avoidance of ice nucleation. The danger of ice nucleation and subsequent ice crystallization leading to frost damage during cooling and rewarming of samples is considered as a critical point of cryopreservation. That is the reason why many of the new progressive methods use and involve a glassy state in plant material intended for cryopreservation. Knowledge of the glass transition temperature is useful not only for improving methods involving glassy state in plant shoot tips. It also provides information essential for the long-term storage of shoot tips.

This biotechnology is based on the induction of the vitrification status – glass induction by dehydration, addition of cryoprotectants and a very fast decrease in temperature. Vitrification can be achieved in a number of ways (Sakai & Engelmann, 2007) but they usually all have the results of increased solute concentration to a critical viscosity. Low water content minimizes the ice crystallization that is potentially dangerous for plant cells and increases the temperature of glass transition. Supposing that the change of water status in the certain range is not limiting for plant regeneration. Plant Vitrification Solutions (PVS) marked with numbers according to the specific mixture of basic cryoprotectants and their concentrations are usually used for osmotic dehydration. Another cryopreservation method used, is based on desiccation in the air-flow cabinet. It is defined with the flow rate, temperature and humidity or on desiccation over various saturated salt solutions with steady-state activity of water.

### 3.1 Cryoprotectants involved in vitrification method

The cryopreservation method using a vitrification solution was first described by (Luyet, 1937). The vitrification solutions were firstly named, according to the first author of the publication and later the vitrification solutions have abbreviated names from Plant Vitrification Solution (PVS) with a number according to the time of their first appearance in the literature. The main ones are Luyet (1937), Fahy (1985), Steponkus (Langis & Steponkus, 1990), PVS1 (Uragami *et al.*, 1989; Towill, 1990), PVS2 (Suzuki *et al.*, 2008), PVS3, PVS4, PVS5 (Nishizawa *et al.*, 1993), VS6 (Liu *et al.*, 2004a), PVSL (Liu *et al.*, 2004b) VSL (Suzuki *et al.*, 2008), with different concentration and combination of the main four components: dimethylsulfoxide, sucrose, glycerol and ethylene glycol. The increased efficiency of vitrification methods was achieved by treating plants in the pre-cultivation step before cryopreservation of plant shoot tips in so called Loading Solution (LS) (Dumet *et al.*, 2002; Matsumoto & Sakai, 1995; Sakai *et al.*, 1991; Sakai & Engelmann, 2007). The cryoprotective substances should fulfil several basic parameters, such as cell permeability, viscosity, toxicity and the minimum concentration necessary for the vitrification, which eliminates the formation of ice crystals.

Cryoprotective substances help to ensure the stability of membranes and enzymes in the subsequent dehydration by vitrification solutions and to avoid the formation of ice crystals (Kantha & Leung, 1979; Kim *et al.*, 2006). The samples are exposed to a several hour-long treatment by some cryoprotective substances, and then they are plunge-frozen in liquid nitrogen. The effect of cryoprotective solution composition for plant regeneration was studied in different plant species (Ellis *et al.*, 2006; Kim *et al.*, 2004; Kim *et al.*, 2009; Tanaka *et al.*, 2004).

In the most recent approaches to the garlic cryopreservation, vitrification method can be induced by treating the shoot tips of plantlets with a highly concentrated a mixture of glycerol and sucrose. (Nishizawa *et al.*, 1993) developed Plant Vitrification Solution 3 (PVS3) with 50% glycerol (w/v) and 50% sucrose (w/v) in water. It is noteworthy that, following these procedures, the plant specimens can be directly plunged into liquid nitrogen, where they can be stored for an indefinite period of time without undergoing the risks of contamination or genetic alterations.

### 3.2 Methods based on dehydration

Potato (*Solanum tuberosum* L.) is a plant species sensitive to frost temperatures. Cryoprotocol for potato has to solve the problem of how to overcome temperature between 0 °C and -130 °C during cooling and warming without ice crystal growth and cell damage. Cold acclimation is not appropriate as pre-cultivation for potato plant (Hirai & Sakai, 1999; Schafer-Menuhr *et al.*, 1996; Kaczmarczyk, 2008). The only method for potato vitrification is a water content decrease in samples, and then the rapid cooling and warming rate. Water content decrease is achieved by preculturing explants with osmotic compounds, air desiccation or vitrification. On bases, vitrification (Sarkar & Naik, 1998), droplet (Schafer-Menuhr *et al.*, 1996) and recently vitrification-droplet (Halmagyi *et al.*, 2004; Schafer-Menuhr *et al.*, 1996) methods were developed or adapted for potato.

### 3.3 Encapsulation-dehydration

One of the other cryopreservation methods is encapsulation-dehydration. The shoot tips were encapsulated in an alginate gel. Experiments with dynamic dehydration studies demonstrated the necessity of meristems encapsulation (Benson *et al.*, 1996; Grospietsch *et*

*al.*, 1999; Hirai & Sakai, 1999). The encapsulation of shoot tips prolongs the dehydration period up to seven hours at a low relative humidity. The alginate beads without shoot tips had approximately the same dehydration-time curve. On the contrary no encapsulated shoot tips were completely dehydrated up to 1 hour. The static dehydration of shoot tips was done over the various saturated salt solutions.

## 4. Cryoprotocols

### 4.1 Garlic

The unripe topsets were surface sterilized by chloramines and 75% ethanol and from this point, all preparations were performed under sterile conditions using sterile instruments and culture media in a laminar flow box. Opening the surface sterilized topset in sterile condition, all the inside structures were sterile (Fig. 1a). The sterile unripe bulblets were removed and cuts were made to the clusters or clumps of 3-8 bulbils. The bulbils varied in the thickness (approximately 2 mm) and in the length (3-5 mm) depending on the genotype and stage of ripening. Inside the unripe bulbils shoot tips were (Fig. 2a) with meristematic tissue.

Pre-culture of unripe clusters of bulbils was done on the MS culture medium (Murashige & Skoog, 1962) with 0,2 mg L<sup>-1</sup> BAP and 0,02 NAA mg L<sup>-1</sup> with 10 % sucrose for 20-24 h at 22 °C and 16 h light in Petri dishes sealed with Parafilm.

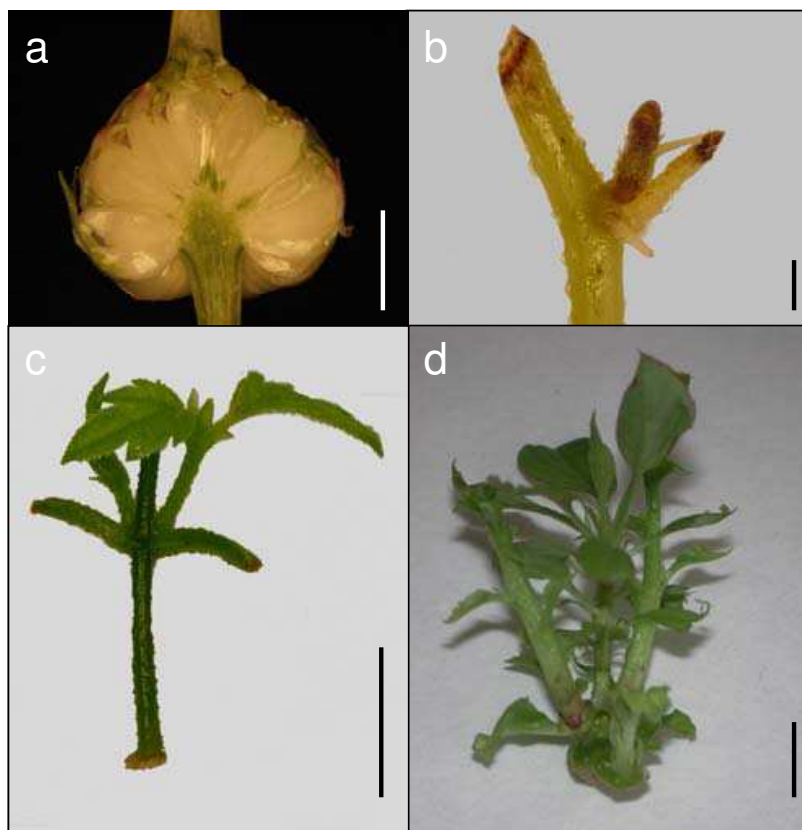


Fig. 1. Plants grown in *in vitro* conditions ready for dissection of shoot tips: (a) Garlic. Scale bar, 5 mm; (b) Potato. Scale bar, 1 mm; (c) Hop. Scale bar, 5 mm and (d) Apple tree. Scale bar, 10 mm.



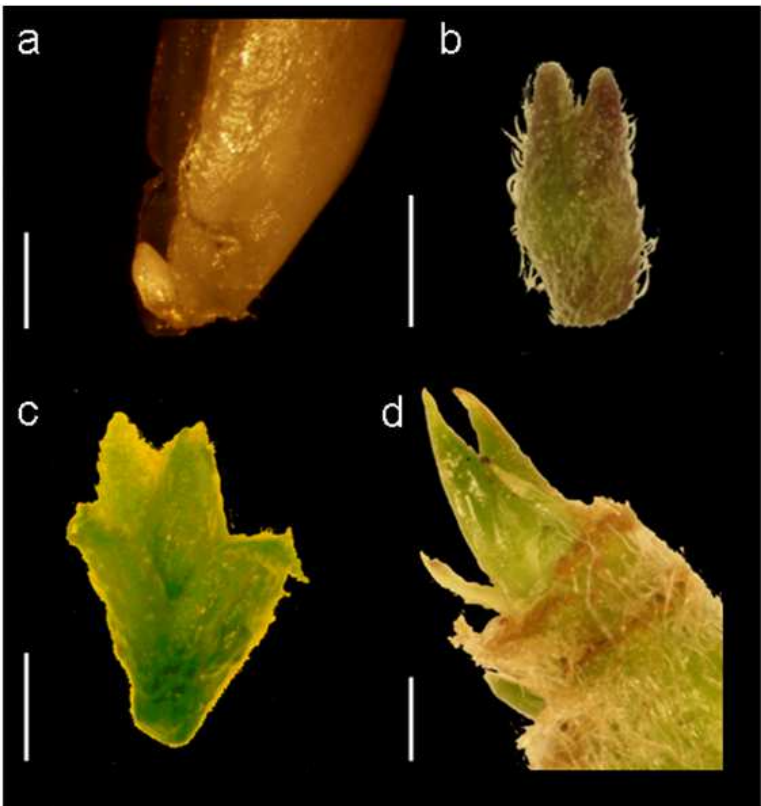


Fig. 2. The size and shape of shoot tips used for cryopreservation. (a) Garlic. Scale bar, 1 mm; (b) Potato. Scale bar, 0,25mm; (c) Hop. Scale bar, 0,25 mm and (d) Apple tree. Scale bar, 1 mm.

Cryoprotocol Steps	The Procedure
Unripe bulbils dehydration	Immersion in the loading solution (13,7 % (w/v) sucrose + 18,4 % (w/v) glycerol in the liquid medium) (Sakai <i>et al.</i> , 1991) for 20 minutes
Cryopreservation	Dehydration by PVS3 (Nishizawa <i>et al.</i> , 1993) at a laboratory temperature for 2 hours Removing and adding fresh PVS3 before freezing Aluminum foil stripes with 5-10 clusters of unripe bulbils plunged directly into liquid nitrogen at least for one hour in liquid nitrogen (Sarkar & Naik, 1998)
Thawing	Rapid warming immersion into a 40 °C water bath for 30-120 seconds for thawing
Survival and regeneration evaluation	Sub-culture on MS medium supplemented with 0,2 mg L <sup>-1</sup> BAP and 0,02 NAA mg L <sup>-1</sup> with 3 % sucrose for seven days in the dark Evaluation of survival after two weeks (Fig. 12a.) Evaluation of regeneration after 8-10 weeks

Table 1. Cryopreservation steps of garlic

4.2 Potato

Potato explants (Fig. 1b.) were multiplied by nodal cuttings in plastic boxes (Vitro Vent container, Duchefa) on 100 ml modified MS medium (Grospietsch *et al.*, 1999) with 7 g L<sup>-1</sup> agar and 30 g L<sup>-1</sup> sucrose, without myo-inositol and phytohormones, with a decreased amount of nitrogen at pH 5,5. Nodal cuttings were cultivated at 22 ± 1 °C, 80 µmol m<sup>-2</sup> s<sup>-1</sup> and photoperiod 16/8 h light/dark (L/D) ( Fig. 1b). Subculture interval was 3-4 weeks.

Nodal cuttings were planted in the same conditions as the pre-cultured plants but only 50 ml medium was used per one box. After 4 day pre-culture, lateral buds elongated to at least 1 mm (Fig. 2b). Subsequently, 25 ml 2 M sucrose was added into each container and explants were cultivated at the same conditions for the next 5-6 days.

Cryoprotocol Steps	The Procedure
Shoot tips dehydration	Shoot tips (Fig. 2b.) (1-2 mm) were transferred onto a filter paper moistened with 14 ml 0,7 M sucrose and phytohormones of the same composition as in a recovery medium (0,5 mg L <sup>-1</sup> IAA + 0,5 mg L <sup>-1</sup> Kinetin + 0.2 mg L <sup>-1</sup> GA3) at 22 ± 1 °C and photoperiod 16/8 h (L/D) and shielded with a leaf of paper for overnight. The second day the shoot tips were transferred (20 tips per foil) onto aluminum foils (20 x 6 x 0.05 mm) Dehydration above silicagel for 1,75 - 2 h.
Cryopreservation	Plunging aluminum foils into liquid nitrogen Stored in cryovials (two foils with 20 shoot tips per vial).
Thawing	Alluminum foils plunged rapidly into the water bath at a laboratory temperature. Transfer immediately onto the recovery medium (Grospietsch <i>et al.</i> , 1999) with the same composition as the pre-culture medium but with phytohormones (0,5 mg L <sup>-1</sup> IAA + 0,5 mg L <sup>-1</sup> Kinetin + 0,2 mg L <sup>-1</sup> GA3)
Survival and regeneration evaluation	Survival was defined by shoot tip growth and by green color and recovery as a new explant development (Fig. 12b.) Plant regeneration was evaluated 2 and 8 weeks after cryopreservation.

Table 2. Cryopreservation steps of potato

4.3 Hop

Maternal plants were cultivated on a multiplication medium without phytohormones at 22 ± 1 °C, 80 µmol m<sup>-2</sup> s<sup>-1</sup>, photoperiod 16/8 h (L/D); subculture interval was 8 weeks. Modified solid medium (Murashige & Skoog, 1962) without casein and myoinositol, with decreased amount of nitrogen (25 % (w/v) of NH<sub>4</sub>NO<sub>3</sub> and 50 % (w/v) of KNO<sub>3</sub> of the original Murashige and Skoog medium), with 40 g L<sup>-1</sup> glucose, pH 5,5 without phytohormones was used as the multiplication medium (MSH).

Nodal cuttings were planted in the same conditions as during explant multiplication but only 50 ml medium was used per one box (Fig 1c). After 7-10 d pre-culture, lateral buds elongated to 1-2 mm. Then the explants were transferred into cold acclimation conditions at 4 °C for 7-10 days. Subsequently 25 ml 0,7 M sucrose was added into each container and explants (Fig. 1c.) were cultivated at the same conditions for the next 7-10 days.

Cryopreservation steps for hop shoot tips were the same as for potato (see Tab. 2).

4.4 Apple tree

*In vitro* plants (Fig. 1d) were cultivated in 100 ml Ehrlenmeyer flasks with 20 ml of MS medium, 3% (w/v) sucrose, 6 g L<sup>-1</sup> agar, supplemented with GA3 1 mg L<sup>-1</sup>, BAP 1 mg L<sup>-1</sup>, IBA 1 mg L<sup>-1</sup>, at 20 ± 1 °C, 8/16 (L/D) photoperiod of light intensity 100 µmol m<sup>-2</sup> s<sup>-1</sup>.

Cryoprotocol Steps	The Procedure
Shoot tips dehydration	One to two week subcultivation on fresh MS medium, Cold hardening for 4-6 weeks at +4± 1 °C, short photoperiod (8/16, L/D) of light intensity 25 µmol m <sup>-2</sup> s <sup>-1</sup> Petri dish with 16 ml of MS medium and poured with 16 ml of 2M sucrose solution for 48 hours Encapsulation of shoot tips (Fig. 2d. ) with meristematic cells in 3% w/v dipped into alginate in 0,75M sucrose for 10 min and then drop into 0,1 M CaCl2 in 0,6 M sucrose for 10 min to net the alginate and form a bead
Cryopreservation	Beads are gently dried on a sterile filter paper Additional dehydration in the laminar flow box at laboratory temperature and dehydrated for different time up to 4 -5 hours, Placed in 2 ml cryovials and plunged in liquid nitrogen.
Thawing	Shoot tips with beads were warmed up by plunging cryovials in 40 °C water
Survival and regeneration evaluation	Survival was defined by shoot tip growth and by green color and regenerated as a new explant development (Fig. 12d.)

Table 3. Cryopreservation steps of apple tree

5. Thermal analysis

Differential scanning calorimetry (DSC) belongs to thermal methods that can be used for measurement and determination of phase and glass transitions for cryopreservation. In principle, the DSC measures the temperatures and heat flows associated with transitions in



plant material as a function of time and temperature. It gives information about endothermic or exothermic changes or changes in heat capacity. The obtained data can be used for determination of glass transition, temperature of ice nucleation, melting, boiling, crystallization time and kinetic reaction – the most important characteristics useful for cryopreservation (Zámečník & Faltus, 2009). The danger of nucleation and subsequent intracellular ice crystallization leading to frost damage during cooling and rewarming of the samples is considered a critical point of plant survival at ultra-low temperatures.

The differential scanning calorimetry method is based on the regulated decrease/increase temperature of the sample and reference and the measurement of temperature and heat flow corresponding to the sample. There are two different types of the differential scanning calorimeters. The power compensation DSC type directly measures heat release/uptake from the sample and the heat flow type measures differences of temperature between reference and sample and recalculates the differential heat flux. The most common cooling/heating rate of the sample is  $10\text{ }^{\circ}\text{C min}^{-1}$ .

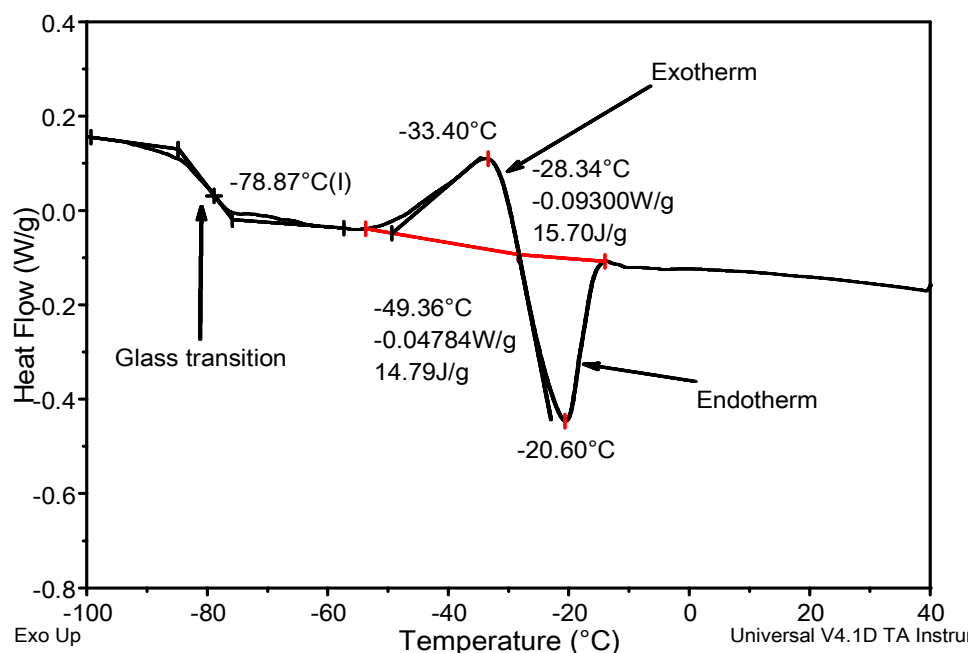


Fig. 3. Example of apple tree shoot tips heat flow response to the temperature. Cooling and warming rate was  $10\text{ }^{\circ}\text{C min}^{-1}$ . Exo up.

In our experiments we used shoot tips of *in vitro* cultures of apple tree. Samples with different water content were obtained by air dehydration of alginate encapsulated shoot tips in the flow box or by dehydration at  $4\text{ }^{\circ}\text{C}$  of *in vitro* cultures. For DSC measurement dissected shoot tips were placed in aluminum sample pans and measured by Differential Scanning Calorimeter TA2920. Samples were cooled down to  $-120\text{ }^{\circ}\text{C}$  (rate of  $10\text{ }^{\circ}\text{C min}^{-1}$ ). The data were collected during heating to  $20\text{ }^{\circ}\text{C}$  (rate of  $10\text{ }^{\circ}\text{C min}^{-1}$ ). The purge gas was either nitrogen or helium.

A Differential Scanning Calorimeter is used as a main tool in cryobiology to assist cryopreservation protocol development, to store thermograms as a documentation of cryo-protocols in the use and to keep information about stored samples and their thermal

properties before, during and after cryopreservation (Benson *et al.*, 1996; Faltus & Zámečník, 2009; Šesták & Zámečník, 2007; Zámečník *et al.*, 2007)

There is an example (Fig. 3) of measured thermal characteristics of shoot tips of apple tree *in vitro* culture cv. Greensleeves by the DSC. Samples of an approximate weight of 10 mg were crimped in an aluminium sample pan and cooled from room temperature to -120 °C. Cooling and heating rate was 10 °C min<sup>-1</sup>. The glass transition, exothermic and endothermic characteristics were analyzed in detail during heating. Thermal characteristics were measured by DSC TA 2920 (TA Instruments) and evaluated by Universal Analysis 2000 for Windows (TA Instruments).

## 6. Water content and glass transition

### 6.1 Garlic

Water content during dehydration of garlic cv. Djambul 2 clusters of shoot tips by PVS3 (Fig. 4). Total amount of water (solid line) and amount of crystallized water (dash line) in shoot tips treated with PVS3 rapidly descend during the first 1,5 hours and further is constant. Crystallized water reaches minimum after 1,75 hours of PVS3 treatment. In this case the decrease of water content in the unripe bulbils is probably so low that it can have no further influence on the glass transition change. In comparison with the measurements in this study on the apple tree shoot tips (see below), the glass transition temperature increases with decreases of water after dehydration.

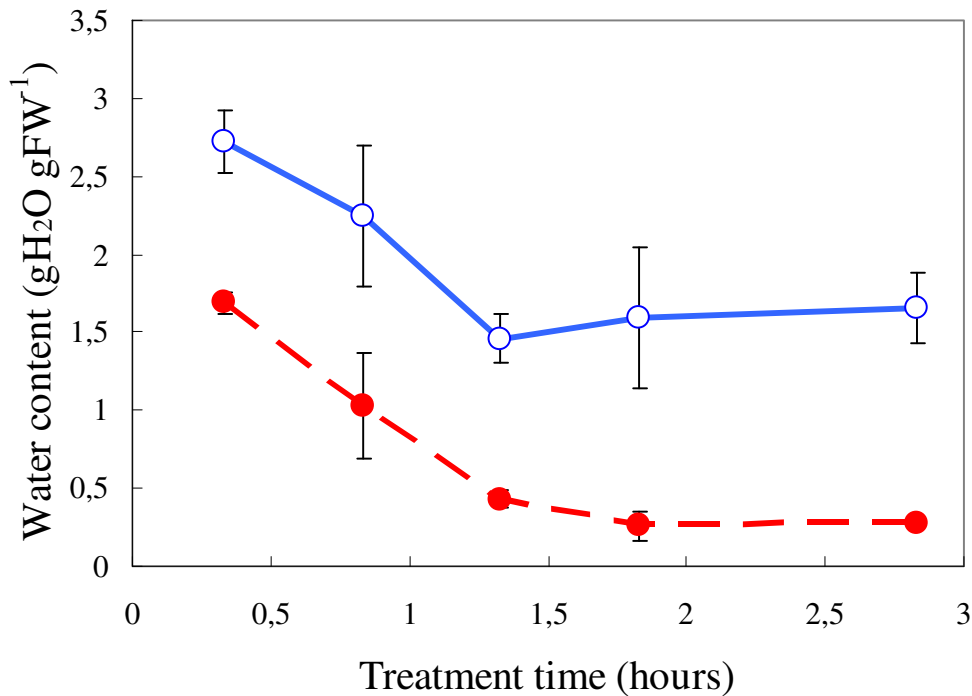


Fig. 4. Unripe garlic bulbils water content (empty circle) and the part of frozen water (full circles) during PVS3 treatment. Note: The unripe bulbils were in the loading solution first 20 minutes than they were immersed in to the PVS3. The bars are standard deviation of mean.

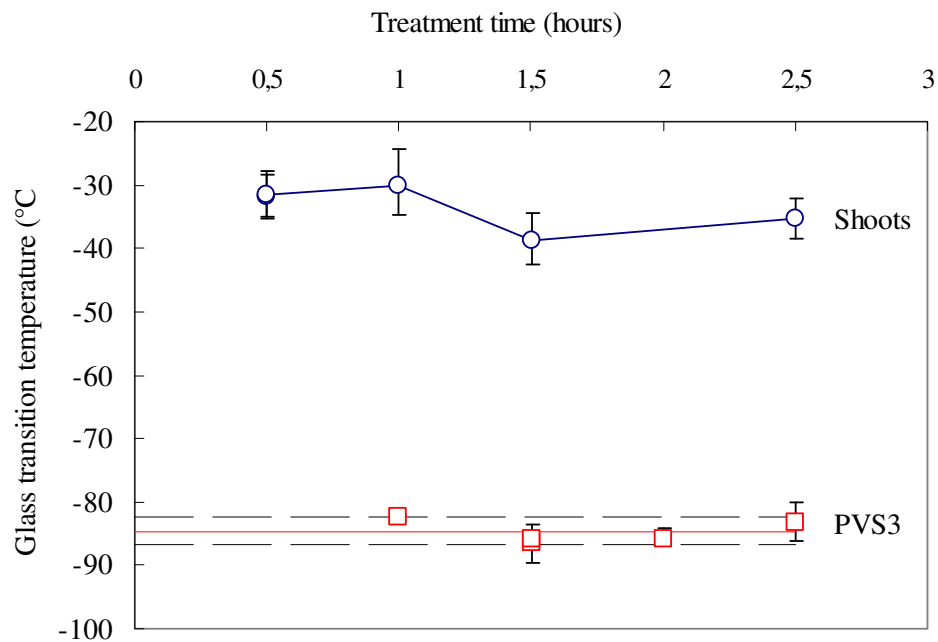


Fig. 5. Glass transition temperature of *Allium* shoot tips after moisture loss by dehydration in the Plant Vitrification Solution 3 (PVS3). Circles show the glass transition midpoint in shoot tips and bars show the onset and endset of glass transition. Squares show the glass transition of PVS3 in shoots. The full line is for the glass transition of PVS3 without shoots. The dashed line above is the endset of glass transition and below the onset of glass transition for PVS3.

Glass transition of garlic shoot tips was measured after different times of treatment – unripe bulbils in PVS3 at 23 °C (Fig. 5). At each curve, there were two S-shape heat flow changes during warming of the samples, typical for glass transition. The lower glass transition temperature on unripe bulbils heat flow curves coincides within the range of onset and endset of the glass transition temperature of PVS3 measured after unloading unripe bulbils. This glass transition temperature can be of PVS3 coating on the surface of the shoot tips immersed in PVS3.

The high glass transition temperature corresponds to glass transition of the shoot tips because at this range of temperature there were no thermal events on the PVS3 temperature dependent curve. There is no significant difference in the change of shoot tip glass transition changes from 0,5 to 2,5 hours of PVS3 treatment. The detectable glass transition was found between -30 °C and -39 °C. The average glass transition temperature is -33,5 °C after 0,5 hour. From these results it is obvious that the glass transition at higher temperature is for shoot tips saturated with PVS3. So, for the survival of shoot tips after thawing from liquid nitrogen, the second glass transition which occurs at higher temperatures is important (Zamecnik *et al.*, 2011).

6.2 Potato

Nodal cuttings were pre-cultured on medium with added sucrose solution. The final sucrose concentration in medium was 0,7 M. The importance of sucrose pre-treatment before potato cryopreservation proved by Grospietsch *et al.*, (1999) and Halmagyi *et al.*,

(2004). Halmagyi *et al.*, (2004) showed the highest plant regeneration after cryopreservation following a pre-treatment with 0,5 M sucrose. Similarly, Sarkar and Naik (1998) found a slightly negative effect of 0,7 M sucrose pre-treatment compared in comparison with 0,5 M or 0,3 M sucrose pre-treatment. In the present study the injury of potato explants was not observed after 0,75 M sucrose treatment.

Total water content in the shoot tips after nodal cutting pre-culture was approximately 5 g of H<sub>2</sub>O per 1 g of dry mass (gH<sub>2</sub>O g DW<sup>-1</sup>) (Fig. 6.). Frozen water content in shoot tips was 4,3 gH<sub>2</sub>O g DW<sup>-1</sup> and the unfrozen 0,7 gH<sub>2</sub>O g DW<sup>-1</sup>. Subsequently shoot tips were isolated and loaded with 0,7 M sucrose in a Petri dish on filter paper for overnight. Total water content of shoot tips decreased to 2,1 gH<sub>2</sub>O g DW<sup>-1</sup>, from which 1,4 gH<sub>2</sub>O g DW<sup>-1</sup> represents the frozen water fraction and 0,7 gH<sub>2</sub>O g DW<sup>-1</sup> the unfrozen water fraction. Because the total water content and frozen water fraction decreased but the unfrozen fraction did not change, the ratio of frozen/unfrozen water content (WC<sub>f</sub>/WC<sub>u</sub>) decreased from 6 to 1,9. The following air dehydration resulted in a decrease of total water content due to both water fractions decrease. After 1,5h air dehydration above silicagel the total water content in shoot tips was 0,49 gH<sub>2</sub>O g DW<sup>-1</sup> from which the frozen water content was 0,09 gH<sub>2</sub>O g DW<sup>-1</sup>, and the unfrozen 0,4 gH<sub>2</sub>O g DW<sup>-1</sup>. Resulting WC<sub>f</sub>/WC<sub>u</sub> ratio decreased to 0,22. The prolonged dehydration decreased both water fractions. After 2h air dehydration above silicagel the total water content in shoot tips was 0,28 gH<sub>2</sub>O g DW<sup>-1</sup> from which 0,006 gH<sub>2</sub>O g DW<sup>-1</sup> belonged to the frozen fraction and 0,276 gH<sub>2</sub>O g DW<sup>-1</sup> to the unfrozen fraction. The WC<sub>f</sub>/WC<sub>u</sub> ratio decreased to 0,12 after 2h air dehydration of shoot tips above silicagel, which represents 2 % crystallized water of the total water content.

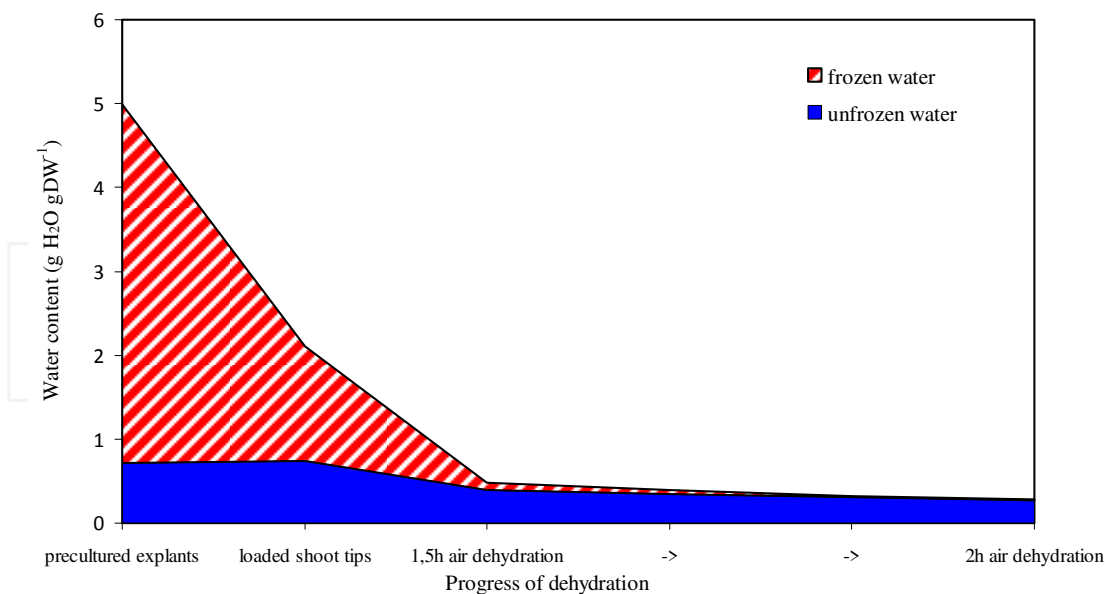


Fig. 6. The progress of dehydration of potato explants (cv. Désirée) after specific steps of cryoprotocol. Explants were pre-cultured on medium with 0,7 M sucrose. The isolated shoot tips were loaded with 0,7 M sucrose solution for overnight. The loaded shoot tips were dehydrated by dry air above silicagel for 2 hours. The amount of frozen and unfrozen water was determined by the DSC analysis.

The decrease in percentage of crystallized water in shoot tips during 1,75 to 2h air dehydration is illustrated in Fig. 6. The crystallized water content decreased from approximately 9% to 2%. Dehydration of shoot tips was connected to the glass transition temperature increase from -38 to -32 °C. The optimal water content of potato shoot tips was approximately 0,4 gH<sub>2</sub>O g DW<sup>-1</sup> that was obtained between 1,5h and 2h air dehydration above silicagel according to the size of particular genotype shoot tips. The temperature of glass transition was approximately -35 °C and the amount of frozen water was very small but still detectable (Fig. 7). Decrease in water content and onset of melting temperature was also found after dehydration by PVS2 solution or 10 % DMSO (Kaczmarczyk, 2008, Kaczmarczyk *et al.* 2011). However the T<sub>g</sub> found by these cryoprotectants was lower than -100 °C. The higher temperature of glass transition found in this study indicated a higher stability of material stored at ultra-low temperatures.

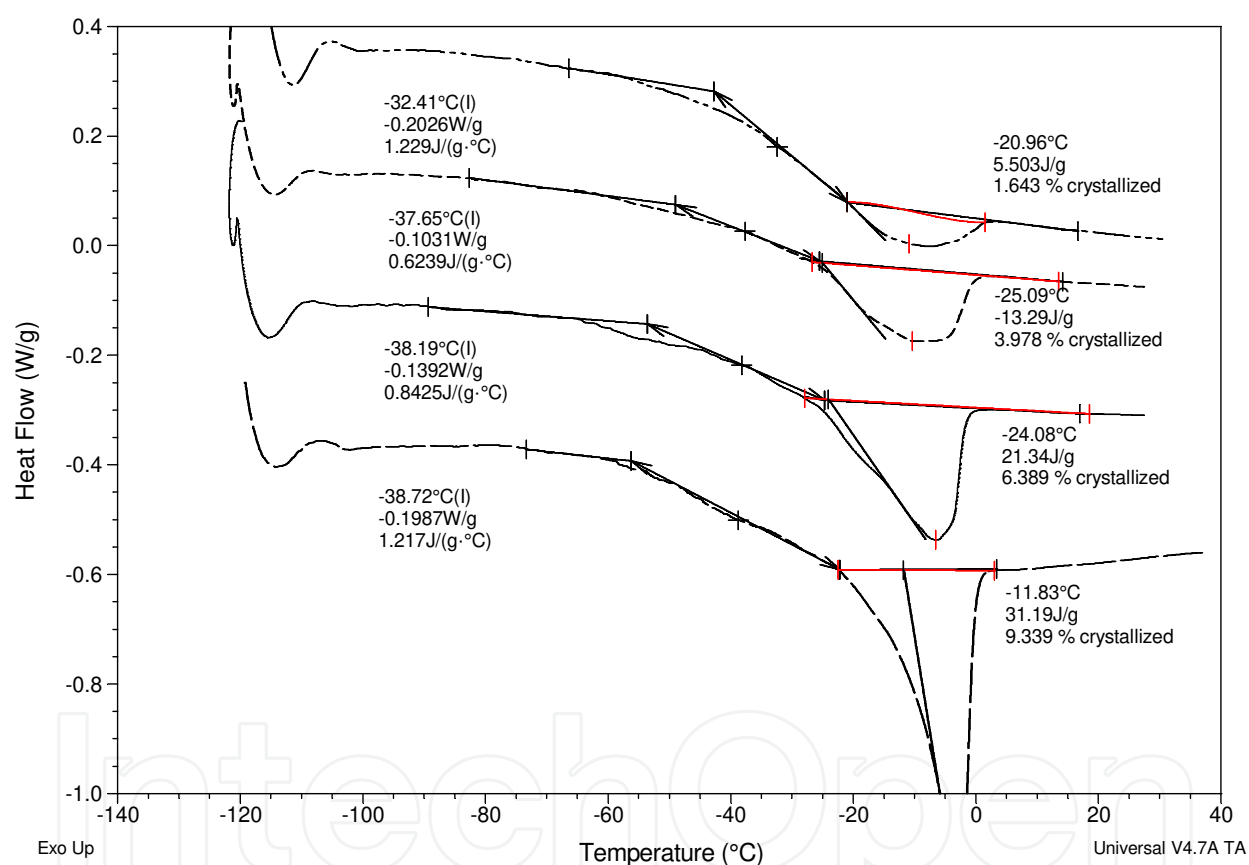


Fig. 7. DSC curves of air dehydrated potato shoot tips (cv. Désirée) air-dehydrated above silicagel for 1,75 to 2 hours. Heat flow was evaluated during warming the samples from -130 to 30 °C by ramp temperature 10 °C min<sup>-1</sup>. Glass transitions were defined by the temperature of glass transition, change of heat flow per g of sample and change in specific heat capacity (C<sub>p</sub>). Melting exotherms are defined by the onset temperature of melting, enthalpy change of thermal event, and crystallinity of water. Curves are shifted along y-axis for clarity according to crystallized water.

The most valuable accessions from sub-collection of old potato cultivars of the Czech origin were selected from the potato in vitro-bank at the Crop Research Institute (CRI) to store them by cryopreservation method. A new cryopreservation method based on nodal cutting



osmotic pre-treatment, shoot tips sucrose loading and their air dehydration on aluminum foils was used for storage of 58 selected potato. All plant accessions prepared for storage in cryo-bank were virus-free. Average post-thaw recovery of hop and potato was 36 % and 25 %, respectively. Recovery of new plants was successful in all tested genotypes.

6.3 Hop

Isolated hop shoot tips (cv. Saazer) were dehydrated by air above silicagel (Fig. 8). Water content was 2,4 g water per 1g dry mass before air dehydration. The highest water decrease was measured during the first 30 minutes of dehydration. Water content of hop shoot tips was 0,68 gH<sub>2</sub>O gDW<sup>-1</sup> after 32 minutes of dehydration. Shoot tips water content decreased below 0,5 gH<sub>2</sub>O gDW<sup>-1</sup> after 70 minutes of dehydration and reached 0,4 gH<sub>2</sub>O gDW<sup>-1</sup> after 100 minutes of dehydration. After 120 minutes the shoot tips water content was 0,37 gH<sub>2</sub>O g DW<sup>-1</sup>. The plant regeneration depended on the time of dehydration, which was influenced by the shoot tips water content. The highest explant regeneration was achieved after 90 minutes of dehydration at a water content close to 0,4 gH<sub>2</sub>O g DW<sup>-1</sup>.

In a former study, a decrease in the endothermic peak was found during air dehydration by encapsulation-dehydration method used for hop cryopreservation (Martinez *et al.*,1998; Martinez *et al.*, 1999; Martinez & Revilla, 1998). A negligible amount of freezable water was detected in shoot tips after the water content decreased to 18 % and no freezable water was found at a water content of 14 %. The glass transition temperature was found at a water content of 18 % and lower. The temperature of glass transition increased with a decrease of water content (Fig. 9).

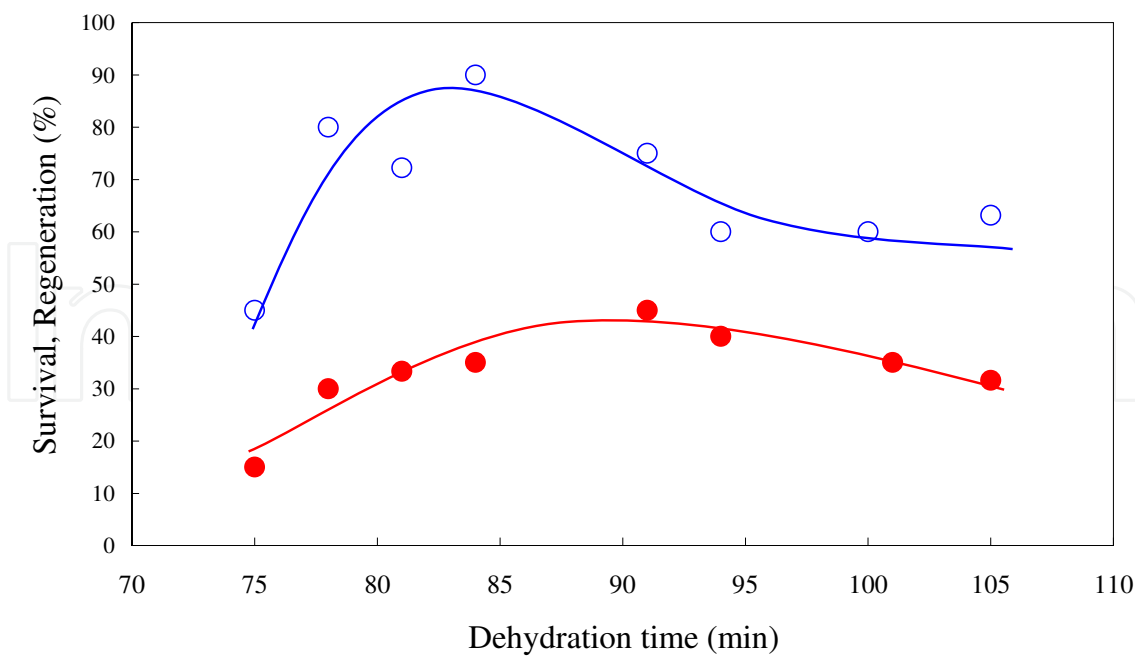


Fig. 8. Survival (empty circles) and regeneration (full circles) of hop explants during dehydration (cv. Saazer).

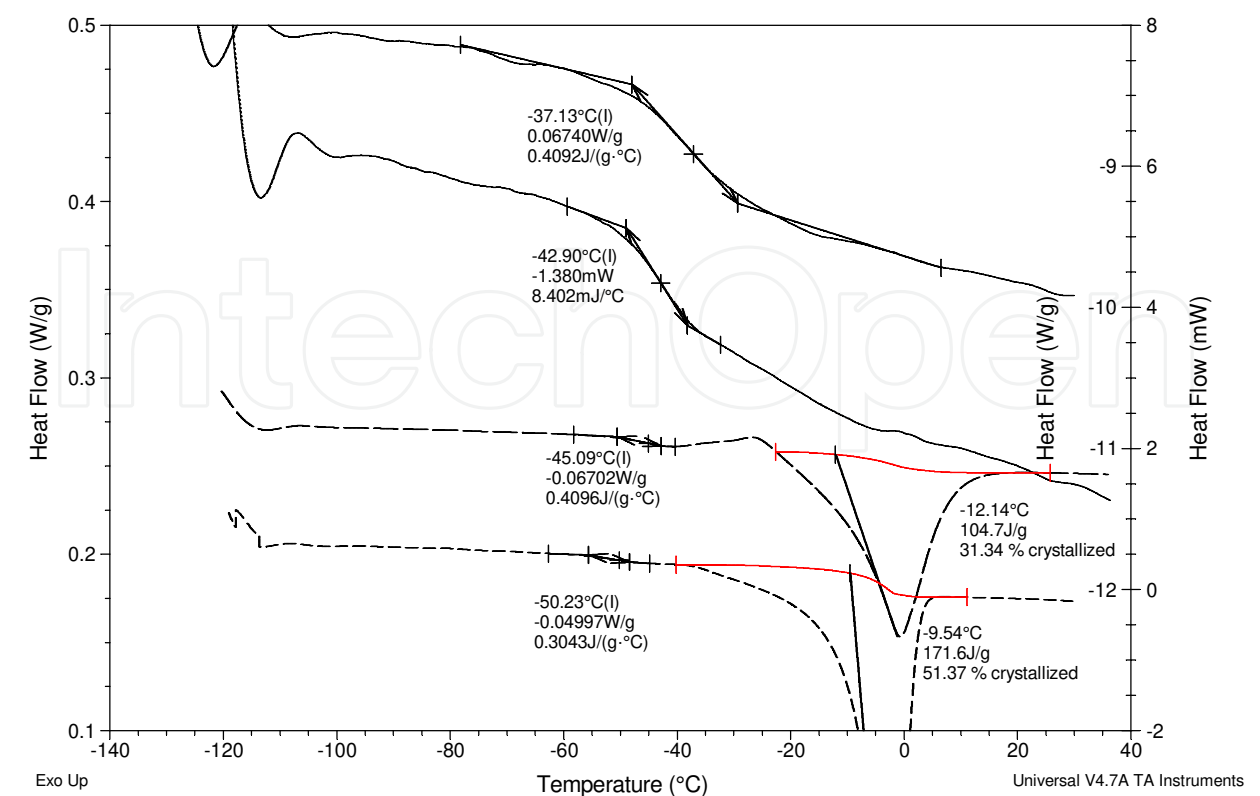


Fig. 9. Heat flow response to the temperature of hop shoot tips. Glass transition temperature of hop shoot tips after moisture loss by dehydration above silicagel. Cooling and warming rate was 10 °C min<sup>-1</sup>. Curves are shifted along y-axis for clarity.

6.4 Apple tree

In Fig. 10, there is an example of measured thermal characteristics of encapsulated shoot tips of apple tree *in vitro* culture cv. Greensleeves by the DSC. Samples of approximate weight of 10 mg were crimped in an aluminium sample pan and cooled from room temperature to -120 °C. The cooling and heating rate was 10 °C min<sup>-1</sup>. The glass transition, exotherm and endotherm characteristics were analysed in detail during heating. Thermal characteristics were measured by DSC TA 2920 (TA Instruments) and evaluated by Universal Analysis 2000 for Windows (TA Instruments).

The course of dehydration of encapsulated shoot tips of *in vitro* cultures of apple tree cv. Greensleeves in an open Petri dish exposed to air flow in laminar flow hood at laboratory temperature is demonstrated in Fig. 10. The determination of water content of 20 encapsulated shoot tips placed in the Petri dish was done by weighing the shoot tips during dehydration after approximately 4 hours of drying to the constant weight in an oven (105 °C). The water content was calculated as a proportion of g of water to g of dry matter. From measurement it was evident, that the dehydration consists of two parts; a faster one at the beginning and a slower one after approximately 2 hours of dehydration. From 2 h of dehydration, the level of water content in encapsulated apple tree shoot tips is almost constant. In Fig. 10, there are thermal characteristics (during heating) of encapsulated apple tree shoot tips during their dehydration in the air flow in flow hood. The more dehydrated the samples, the higher the glass transition temperature (characterised by the inflex point I)

was measured and no endothermal events representing water in ice crystal form were detected below 0,4 gH<sub>2</sub>O g DW<sup>-1</sup>. The value of 0,4 gH<sub>2</sub>O g DW<sup>-1</sup> dehydration level corresponds to the levels recommended also by other authors (Gupta & Reed, 2006; Martinez *et al.*, 1999; Wu *et al.*, 1999). The integration of endotherm areas of shoot tip and alginate confirms the importance of dehydration to the levels when ice crystals are not present in shoot tip tissues (Figs. 10,11). The energy counted as integration of the endothermic peak corresponded to the amount of frozen water; the less energy, the smaller amount of ice crystals in the sample. These thermal results led us to dehydrating encapsulated shoot tips below 0,4 gH<sub>2</sub>O gDW<sup>-1</sup>.

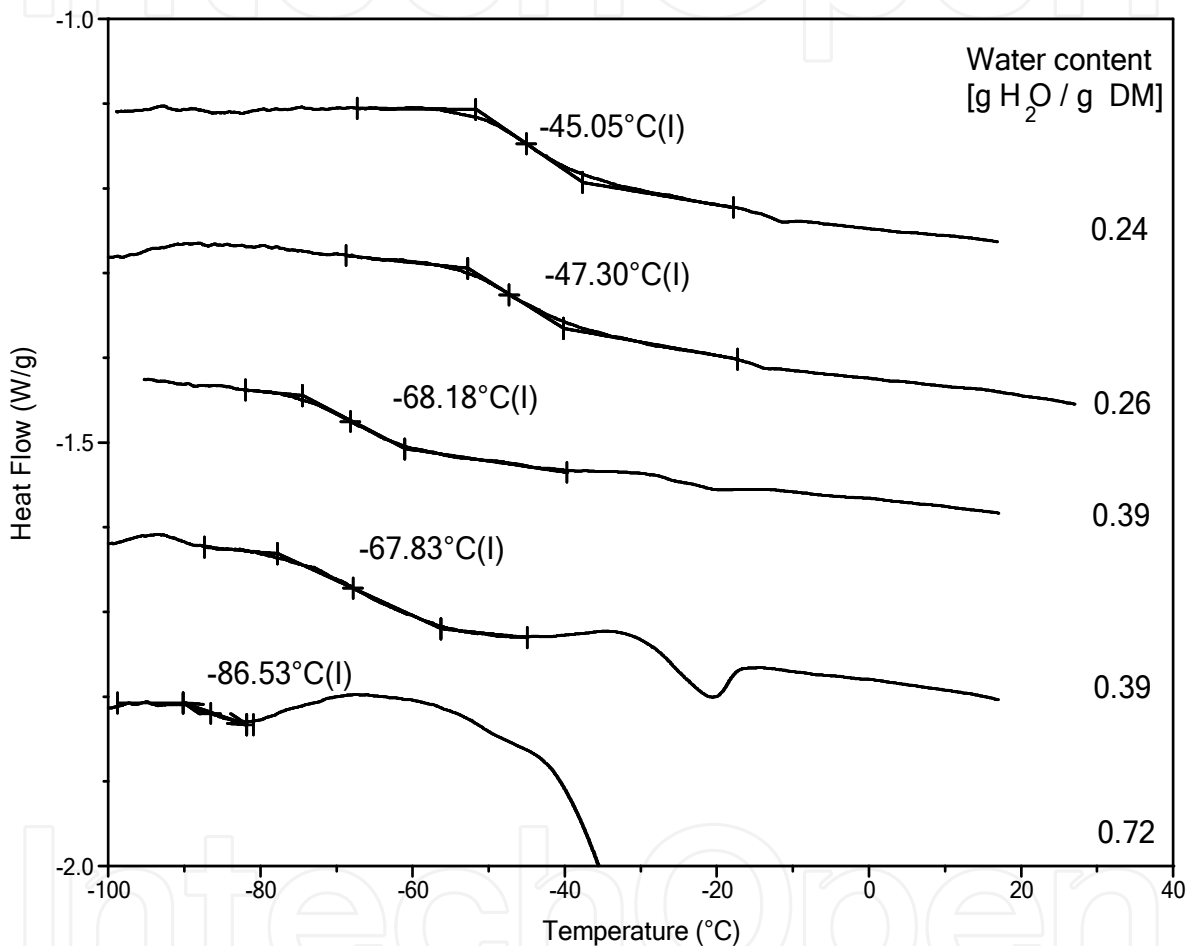


Fig. 10. Glass transition temperature as an inflection point of heat flow change of encapsulated apple tree shoot tips after water loss expressed as final water content (figures behind the end of the separate curves) by dehydration in the air flow. Curves are shifted along y-axis for clarity.

Dehydration curves corresponding to the loss of water from encapsulated *in vitro* shoot tips were measured (Figs. 10,11). During the dehydration procedure of cryopreservation The proper time/level of dehydration must be taken into consideration for successful cryopreservation.

The less water in plant tissues, the less probable damage from ice crystal formation and growth. On the other side plant tissues withstand only certain dehydration. The most

appropriate level of dehydration is determined by DSC by measurement of frozen and unfrozen water (generally it is possible to say water in glassy state) (Fig. 11). After 4h dehydration of encapsulated *in vitro* shoot tips the water content decreased below 0,3 gH<sub>2</sub>O gDW<sup>-1</sup>. Water content decrease slows down markedly at the level of 0,6 gH<sub>2</sub>O gDW<sup>-1</sup>. From this level of dehydration, both exotherms and endotherms start to disappear which corresponded to the end of ice crystal formation and the start of glass transitions with high change of heat capacity (Fig. 11). The survival and regeneration of cryopreserved apple tree shoot tips, cultivar Greensleeves, were 75 % and 53 % respectively after 4h dehydration. Non-dehydrated shoot tips neither survived nor regenerated. Dehydration of shoot tips to the level of glass formation is a crucial factor for their survival at ultralow temperatures.

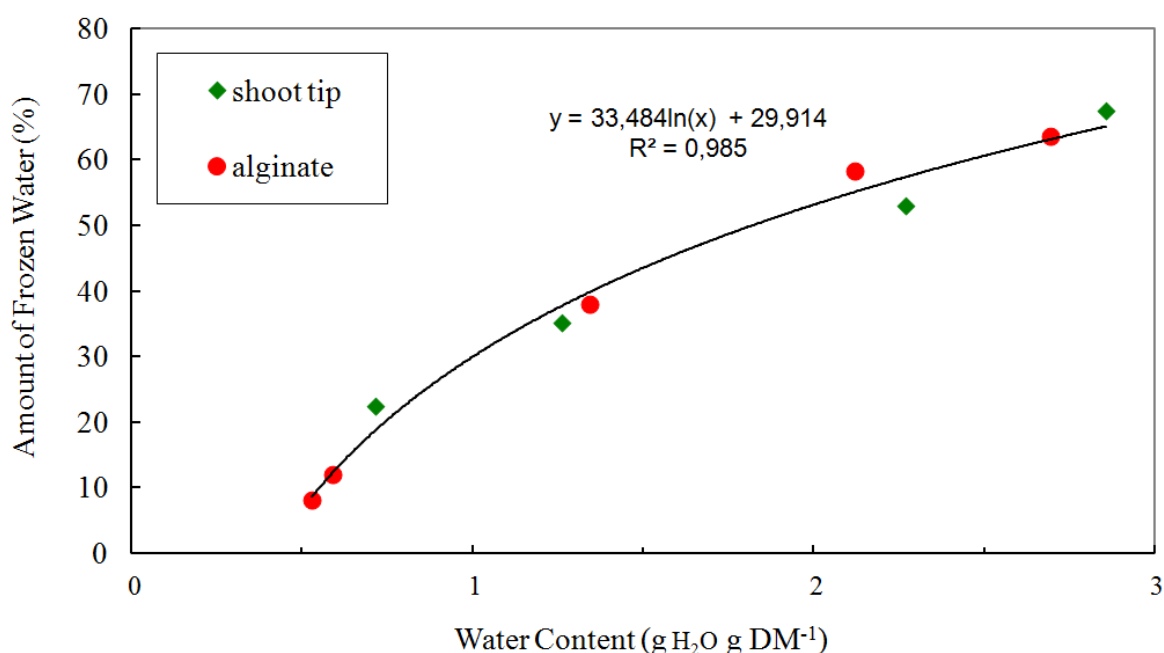


Fig. 11. The amount of frozen water of apple tree shoot tips and alginate beads during dehydration was as the integration of endotherm areas of shoot tip and alginate. Samples of an approximate weight of 3-10 mg were crimped in an aluminum sample pan and cooled from room temperature to -120 °C. The cooling and heating rate was 10 °C min<sup>-1</sup>. The enthalpy counted as an integration of the endothermic peak corresponded to the amount of frozen water; the less enthalpy the smaller amount of ice crystals in the sample.

## 7. Regeneration of plants after cryopreservation

The regeneration rate of unripe garlic bulbils was close to 100 % in comparison with the lower regeneration rate of ripe bulbils. The results for ripe bulbils were done on 173 accessions (the measurements on ripe bulbils were not presented, Grospietsch unpublished). The average regeneration rate of ripe bulbils was 40 % and unripe bulbils near 100%. The optimized droplet-vitrification protocol was successfully applied to bulbil primordia of garlic varieties also with high regeneration percentages ranging between 77,4-95% (Engelmann, 2011; Kim *et al.*, 2006)

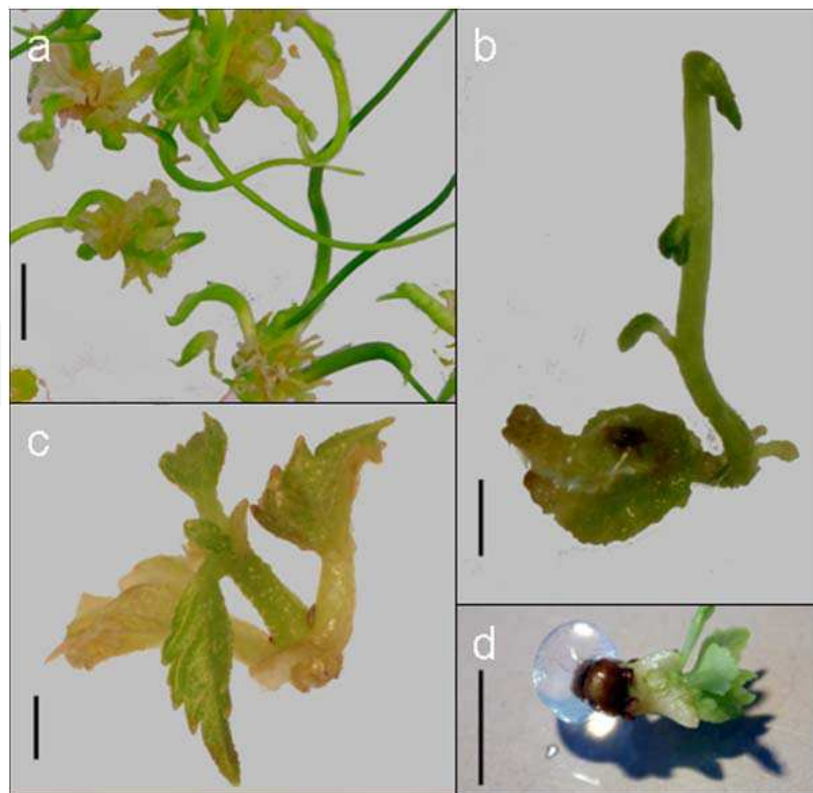


Fig. 12. Plants regenerated into new plants after immersion in and thawing from the liquid nitrogen: (a) Garlic. Scale bar, 5 mm; (b) Potato. Scale bar, 2 mm; (c) Hop. Scale bar, 1 mm and (d) Apple tree. Scale bar, 5 mm.

The average recovery after cryopreservation of fifty potato cultivars was 24,8% and average hop recovery was 30,5%. Plant recovery was improved due to the cryoprotocol and media modifications and the average recovery of potato and hop in the year 2007 was 29,1% and 35,5%, respectively (Fig. 13). The highest frequency of plant recovery was near to the average recovery in both crops.

To improve the stability and safety of potato collection, the cryo-collection of the Czech potato germplasm was established. The sub-collection of old potato varieties of the Czech origin was selected as the most important part of potato germplasm kept in the Czech In Vitro Bank of Potato. Fifty eight selected genotypes were cryopreserved by a new method based on osmotic adjustment of explants with sucrose and following air-dehydration. Currently these 58 old potato cultivars of the Czech origin were backed up in cryo-collection at the CRI in Prague.

The differences in plant survival and regeneration exist either among species or cultivars. Example of survival and regeneration of apple tree *in vitro* cultures cryopreserved by the encapsulation-dehydration method are shown in (Tab. 4). The differences can be caused either by different reaction of cultivars in cryopreservation protocol or cold hardening conditions or *in vitro* cultivation. For example, the cultivar McIntosh belongs to very cold resistant cultivars and also has very high regeneration after cryopreservation. On the contrary, the very cold tender cultivar Zvonkové had no survival in laboratory frost tolerance test on dormant buds (data not shown) but *in vitro* cultures were able to survive the cryopreservation procedure, although with very weak regeneration.



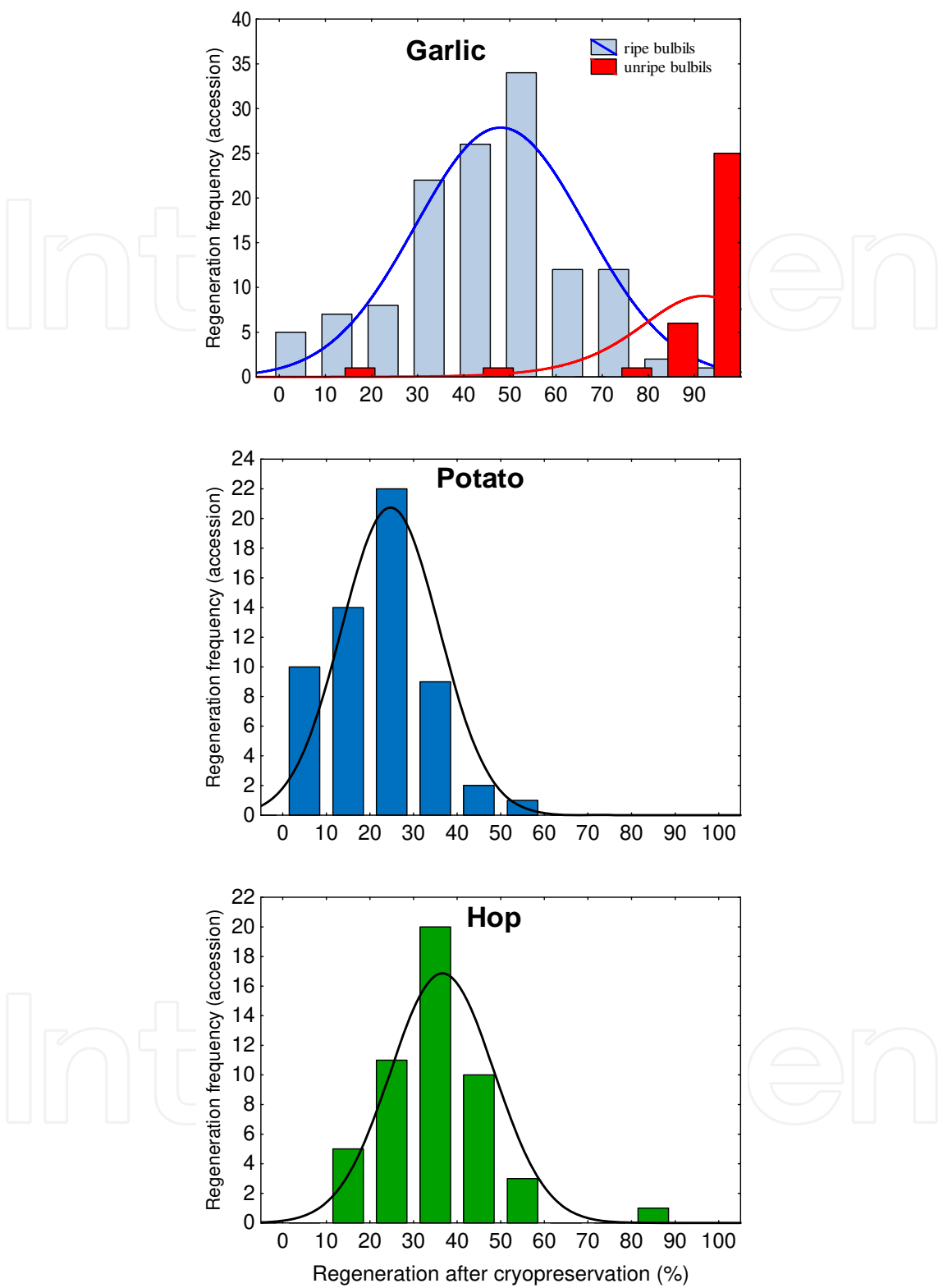


Fig. 13. Regeneration frequency of genotypes (accessions) of garlic, potato and hop stored in the Cryobank of vegetatively propagated crops. Altogether, 129 accessions were evaluated in ripe garlic bulbils, 34 accessions in unripe garlic bulbils, 58 accessions in potato and 50 in hop.

There is clear evidence for the necessity of physiological and biochemical adaptations of cryopreservation procedures according to the different demands of used cultivars to fulfill the needs for successful cryopreservation of apple tree *in vitro* germplasm.

Apple tree cultivar	Survival [%]	SD	Regrowth [%]	SD	n	Number of freezing
Alkmene	6 <sup>a</sup>	0,4	6 <sup>a</sup>	0,4	32	2
Golden Delicious	75 <sup>bc</sup>	27,9	55 <sup>bcd</sup>	24,7	39	3
Greensleeves	75 <sup>bc</sup>	15,0	53 <sup>bcd</sup>	7,5	30	2
Chodské	63 <sup>bc</sup>	17,6	46 <sup>abcd</sup>	13,8	84	5
Idared	34 <sup>abc</sup>	13,5	34 <sup>abc</sup>	13,5	45	2
Jonagold	63 <sup>bc</sup>	13,4	44 <sup>abcd</sup>	26,2	50	3
McIntosh	85 <sup>c</sup>	15,0	85 <sup>d</sup>	15,0	20	2
Prima	28 <sup>ab</sup>	15,0	21 <sup>ab</sup>	8,0	20	2
Rubín	78 <sup>bc</sup>	11,0	78 <sup>cd</sup>	11,0	32	2
Zvonkové	44 <sup>abc</sup>	18,7	4 <sup>a</sup>	5,4	56	4
Average	75	21,7	43	21,2	41	3

<sup>a-d</sup> average followed by the same index did not significantly differ at  $P < 0,05$  (analysis of variance – Duncan’s test) SD - standard deviation ( $P < 0,05$ ).

Table 4. Survival and regrowth of apple tree cultivars after encapsulation–dehydration cryopreservation protocol. (n = total number of shoot tips used for evaluations of regeneration).

*In vitro* shoot tips of apple tree were cryopreserved also by vitrification (PVS2) and pre-culture dehydration methods, but the results were not adequate (Tab. 5). Thus the basic approach of cryopreservation of fruit trees in our laboratory was the encapsulation-dehydration method. The average survival and regeneration of evaluated apple tree cultivars were 75 % and 53 %, respectively (Tab. 4). Similar values of regeneration were obtained by (Condello *et al.*, 2011) with the droplet-vitrification method in two apple tree cultivars. On the other hand, (Wu *et al.*, 2001) reached regeneration of up to 86 %. They recommended a prolonged subcultivation of mother plants and their cold acclimation, which decreased water content in shoot tips and subsequently increased the regeneration in all cryopreservation procedures they evaluated. According to our unpublished data and in concordance with other authors, (Wu *et al.*, 2001; Condello *et al.*, 2011), the adaptation of the *in vitro* mother plants appears to be one of the important steps for improving regeneration of cryopreserved cultivars with lower survival. The encapsulation-dehydration cryopreservation method is a suitable tool to conserve a broader spectrum of apple tree germplasm.

8. Comparison of different cryopreservation methods

The bases for achieving high regeneration after plant cryopreservation using a vitrification method needs to observe the time fulfilment in dehydrating procedures and prevention of

tissue damage by chemical toxicity of the cryoprotectants. Otherwise, plant parts in solutes can be injured by the cryoprotectant, by strong osmotic stress during the cryoprotective solution treatment. The time for a high regeneration rate of plants after cryopreservation must be optimized. During this time the explants are dipped in the vitrification solutions, at the optimal temperature, which is involved in the procedure (Condello *et al.*, 2011; Faltus & Zamecnik, 2009; Sakai & Engelmann, 2007; Zámečník *et al.*, 2007).

The temperature-induced glasses-the point at which this occurs, is called the glass transition temperature ( $T_g$ ) - molecular motion nearly ceases and the liquid becomes a glassy solid. Vitrification of cells and tissues is a physical process which avoids intracellular ice crystallization during ultra-rapid freezing by the transition of the aqueous solution of the symplast into an amorphous glassy state of the cells. As a consequence of the vitrification process, plant tissues are protected from the damage and remain viable during their long-term storage at  $-196\text{ }^{\circ}\text{C}$ .

Experimental determination of glassy state in plants is complicated by the endothermic reaction overlapping with the glass transition. Ice crystallization as the first-order reaction has discontinuous change in heat capacity contrary to glass transition, which is characterized by heat capacity change. The main problem is to distinguish the endothermic reaction and the glass transition temperature during the measurement of the thermal events. Thermal analysis methods of glass transition temperature and temperature of ice crystals melting in plant tissues were determined. Standard Differential Scanning Calorimetry (DSC) method and Temperature Modulated Differential Scanning Calorimetry (TMDSC) were usually used (Condello *et al.*, 2011; Zámečník & Faltus, 2009).

Plant	Cryopreservation Method		
	Droplet -vitrification (PVS3)	Ultra-rapid freezing	Encapsulation -dehydration
Garlic	**	NT	*
Potato	*	**	*
Hop	NT	**	NT
Apple tree	*(§)	*	**

Table 5. Cryopreservation methods used in this study. Results obtained by cryopreservation methods with a high regeneration rate were presented only: \*\* - high regeneration rate; \* - tested; NT- not tested; (§)-PVS2 was used.

The vitrification method, based on involvement of biological glass, requires a highly concentrated solution of cryoprotectant (from 5 to 8 M), at which the cells are osmotically dehydrated to a certain level. This level of dehydration is characterized by no frost-heaving of water and with a minimum, or no production of ice crystals. The cells treated by cryoprotectant are then vitrified before or during immersion into liquid nitrogen (Sakai *et al.*, 1991). Vitrification belongs to new well-developed procedures supplying frost dehydration of cells pursued at a low temperature. Namely, it removes most of the freezable water through the exposure of the plant shoot tips to a highly concentrated vitrification solution at temperatures above the freezing point.

Several procedures of cryopreservation of fruit trees germplasm, especially apple tree were evaluated, including two-step cryopreservation with controlled ice nucleation (Niino *et al.*, 1992; Niino & Sakai, 1992; Seufferheld *et al.*, 1999; Zhao *et al.*, 1999), and the type of vitrification method of extirpated *in vitro* cultures. The procedures were adjusted and corrected according to the thermal methods for determination of ice nucleation (Tyler *et al.*, 1988), glass transitions in plant material, and exothermic and endothermic characteristics of plant buds. A combination of encapsulation-dehydration cryoprotocol was chosen as the most appropriate system of *in vitro* cultures (Chang & Reed, 2001). The reaction of selected cultivars was different on *in vitro* sub-cultivation and subsequent cryopreservation protocol.

Many plants from the temperate and tropical region were successfully cryopreserved by the encapsulation-dehydration method, which belongs to cryopreservation methods. The encapsulation-dehydration cryopreservation procedure is based on encapsulating shoot tips of pretreated *in vitro* plants with subsequent dehydration either in sterile air flow (Benson *et al.*, 1996; Dereuddre *et al.*, 1991) or above silicagel (Grospietsch *et al.*, 1999). Dehydrated beads with encapsulated shoot tips are in most cases plunged directly into liquid nitrogen or slowly frozen in programmable freezers (Fabre & Dereuddre, 1990; Zhao *et al.*, 2001). Rewarming proceeds either slowly by placing beads on Petri dishes or the cryotubes are placed in a water bath of temperature ranging from 25 to 45 °C for several minutes (Gupta & Reed, 2006; Matsumoto & Sakai, 1995). Survival and regeneration of shoot tips are evaluated after placing the re-warmed beads on cultivation medium which can be compound modified by phytohormones (their combination and concentration), to stimulate proliferation or eliminate the phenolic compounds (Paulet *et al.*, 1993). The medium can be softer in some cases to allow an easier regeneration from beads (Reed *et al.*, 2006) or even the explants can be extracted from the beads (Niino & Sakai, 1992). The importance of direct explant regeneration to the new plants without callus inter phase is important for the genetic stability after usage of this method.

## 9. Conclusion

The four main crops (garlic, potato, hop and apple tree) have been cryopreserved in the Czech Plant Cryobank. The methods of cryopreservation are based on cryoprotocol of the vitrification procedures (encapsulation-dehydration, dehydration by vitrification solution and a modified ultra-rapid freezing method based on preconditioning of the plant shoot tips on an osmotic solution). Cryopreservation is well advanced for vegetatively propagated species, and techniques are ready for large-scale experimentation in an increasing number of cases (Engelmann, 2011). We have started the routine cryopreservation with Czech potato genotypes in collaboration with Potato Research Institute Ltd., Havlíčkův Brod. The other three crops are supported by specialized companies (*Allium* genera by the Gene Bank Olomouc branch, the Research and Breeding Institute of Pomology at Holovousy and the Hop Research Institute). The Czech Plant Cryobank operates as a safe duplicate to repositories of germplasm kept in field or *in vitro* conditions. In this way the Czech Plant Cryobank in Prague joined the effort of the potato cryobank in Germany (Keller *et al.*, 2008), and Korea (Kim *et al.*, 2006). Currently three of the EU countries (The Czech Republic, Germany and Poland) involved in the maintenance of national vegetatively propagated *Allium* collections are developing the methodology for cryopreservation (project

EURALLIVEG, EU) and established the Tripartite *Allium* Cryobank to store meristematic explants in liquid nitrogen.

## 10. Acknowledgment

This work was partially supported by project 0002700604 MSMT and QH71228 MZe. The authors would like to thank M. Grospietsch, Jana Chittendenová, Aneta Babická and Adéla Hreňuková for their help and Dr. V. Skládál for critical review of this manuscript.

## 11. References

- Benson, E.B.; Reed, B.M.; Brennan, R.M.; Clacher, K.A. & Ross, D.A. (1996). Use of thermal analysis in the evaluation of cryopreservation protocols for *Ribes nigrum* L germplasm. *Cryo-Letters*, Vol.17, No.6, (November 1996), pp. 347-362, ISSN0143-2044
- Benson, E.E. (2008). Cryopreservation of phytodiversity: A critical appraisal of theory practice. *Critical Reviews in Plant Sciences*, Vol.27, No.3, (2008), pp. 141-219, ISSN0735-2689
- Chang, Y.J. & Reed, B.M. (2001). Preculture conditions influence cold hardiness and regrowth of *Pyrus cordata* shoot tips after Cryopreservation. *Hortscience*, Vol.36, No.7, (December 2001), pp. 1329-1333, ISSN0018-5345
- Condello, E.; Caboni, E.; Andre, E.; Piette, B.; Druart, P.; Swennen, R. & Panis, B. (2011). Cryopreservation of Apple in Vitro Axillary Buds Using Droplet-Vitrification. *Cryoletters*, Vol.32, No.2, (March 2011), pp. 175-185, ISSN0143-2044
- Dereuddre, J.; Hassen, N.; Blandin, S. & Kaminski, M. (1991). Resistance of Alginate-Coated Somatic Embryos of Carrot (*Daucus-Carota* L) to Desiccation and Freezing in Liquid-Nitrogen .2. Thermal-Analysis. *Cryo-Letters*, Vol.12, No.3, (May 1991), pp. 135-148, ISSN0143-2044
- Dumet, D.; Grapin, A.; Bailly, C. & Dorion, N. (2002). Revisiting crucial steps of an encapsulation/desiccation based cryopreservation process: importance of thawing method in the case of *Pelargonium* meristems. *Plant Science*, Vol.163, No.6, (December 2002), pp. 1121-1127, ISSN0168-9452
- Ellis, D.; Skogerboe, D.; Andre, C.; Hellier, B. & Volk, G. (2006). Implementation of garlic cryopreservation techniques in the national plant germplasm system. *Cryoletters*, Vol.27, No.2, (March 2006), pp. 99-106, ISSN0143-2044
- Engelmann, F. (2011). Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular & Developmental Biology-Plant*, Vol.47, No.1, (February 2011), pp. 5-16, ISSN1054-5476
- Fabre, J. & Dereuddre, J. (1990). Encapsulation Dehydration - A New Approach to Cryopreservation of *Solanum* Shoot-Tips. *Cryo-Letters*, Vol.11, No.6, (November 1990), pp. 413-426, ISSN0143-2044
- Fahy, G.M. (1985). Cold Shock Injury Is A Significant Factor in Freezing-Injury - A Position Against. *Cryobiology*, Vol.22, No.6, (1985), pp. 628-628, ISSN0011-2240
- Faltus, M. & Zámečník, J. (2009). Thermal Characteristics of Some Vitrification Solutions. *Cryoletters*, Vol.30, No.5, (September 2009), pp. 389-389, ISSN0143-2044



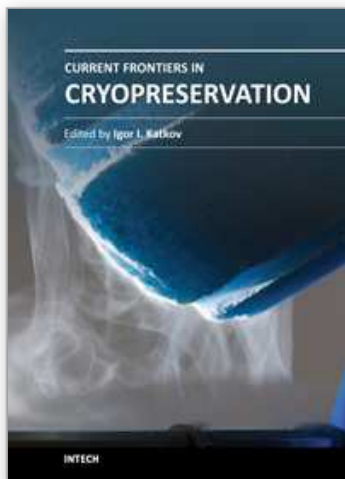
- Gonzalez-Arnan, M.T.; Panta, A.; Roca, W.M.; Escobar, R.H. & Engelmann, F. (2008). Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. *Plant Cell Tissue and Organ Culture*, Vol.92, No.1, (January 2008), pp. 1-13, ISSN0167-6857
- Grospietsch, M.; Stodůlková, E. & Zámečník, J. (1999). Effect of osmotic stress on the dehydration tolerance and cryopreservation of *Solanum tuberosum* shoot tips. *Cryo-Letters*, Vol.20, No.6, (November 1999), pp. 339-346, ISSN0143-2044
- Gupta, S. & Reed, B.M. (2006). Cryopreservation of shoot tips of blackberry and raspberry by encapsulation-dehydration and vitrification. *Cryoletters*, Vol.27, No.1, (January 2006), pp. 29-42, ISSN0143-2044
- Halmagyi, A.; Fischer-Kluver, G.; Mix-Wagner, G. & Schumacher, H.M. (2004). Cryopreservation of *Chrysanthemum morifolium* (*Dendranthema grandiflora* Ramat.) using different approaches. *Plant Cell Reports*, Vol.22, No.6, (January 2004), pp. 371-375, ISSN0721-7714
- Hirai, D. & Sakai, A. (1999). Cryopreservation of in vitro-grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-vitrification. *Potato Research*, Vol.42, No.2, (1999), pp. 153-160, ISSN0014-3065
- Kaczmarczyk, A. (2008). Physiological, biochemical, histological and ultrastructural aspects of cryopreservation in meristematic tissue of potato shoot tips (2008), ISSN 978-3-89574-694-9
- Kaczmarczyk, A., Rokka, V-M & Keller E. R. J. (2011). Potato Shoot Tip Cryopreservation. A Review Source: *Potato research*, Vol. 54, No. 1, (March 2011), pp. 45-79, ISSN0014-3065
- Kartha, K.K. & Leung, N.L. (1979). Cryopreservation of Plant Meristems As A Means of Germ Plasm Storage. *Cryobiology*, Vol.16, No.6, (1979), pp. 582-583, ISSN0011-2240
- Keller, E.R.J.; Kaczmarczyk, A. & Senula, A. (2008). Cryopreservation for plant genebanks - A matter between high expectations and cautious reservation. *Cryoletters*, Vol.29, No.1, (January 2008), pp. 53-62, ISSN0143-2044
- Kim, H.H.; Cho, E.G.; Baek, H.J.; Kim, C.Y.; Keller, E.R.J. & Engelmann, F. (January 2004). Cryopreservation of garlic shoot tips by vitrification: Effects of dehydration, rewarming, unloading and regrowth conditions. *Cryoletters*, Vol.25, No.1, (January 2004), pp. 59-70, ISSN0143-2044
- Kim, H.H.; Lee, J.K.; Yoon, J.W.; Ji, J.J.; Nam, S.S.; Hwang, H.S.; Cho, E.G. & Engelmann, F. (2006). Cryopreservation of garlic bulbil primordia by the droplet-vitrification procedure. *Cryoletters*, Vol.27, No.3, (May 2006), pp. 143-153, ISSN0143-2044
- Kim, H.H.; Lee, Y.G.; Shin, D.J.; Ko, H.C.; Gwag, J.G.; Cho, E.G. & Engelmann, F. (2009). Development of Alternative Plant Vitrification Solutions in Droplet-Vitrification Procedures. *Cryoletters*, Vol.30, No.5, (September 2009), pp. 320-334, ISSN0143-2044
- Langis, R. & Steponkus, P.L. (March 1990). Cryopreservation of Rye Protoplasts by Vitrification. *Plant Physiology*, Vol.92, No.3, (March 1990), pp. 666-671, ISSN0032-0889
- Liu, H.Q.; Yu, W.G.; Dai, J.X.; Gong, Q.H.; Yang, K.F. & Lu, X.Z. (2004a). Cryopreservation of protoplasts of the alga *Porphyra yezoensis* by vitrification. *Plant Science*, Vol.166, No.1, (January 2004a), pp. 97-102, ISSN0168-9452

- Liu, Y.G.; Wang, X.Y. & Liu, L.X. (2004b). Analysis of genetic variation in surviving apple shoots following cryopreservation by vitrification. *Plant Science*, Vol.166, No.3, (2004b), pp. 677-685, ISSN0168-9452
- Luyet, B. (1937). On the mechanism of cellular death by high pressure cytological modifications accompanying death in yeast. *Comptes Rendus Hebdomadaires Des Seances De l'Academie Des Sciences*, Vol.204, (1937), pp. 1506-1508, ISSN0001-4036
- Martinez, D. & Revilla, M.A. (1998). Cold acclimation and thermal transitions in the cryopreservation of hop shoot tips. *Cryo-Letters*, Vol.19, No.6, (November 1998), pp. 333-342, ISSN0143-2044
- Martinez, D.; Revilla, M.A.; Espina, A.; Jaimez, E. & Garcia, J.R. (1998). Survival cryopreservation of hop shoot tips monitored by differential scanning calorimetry. *Thermochimica Acta*, Vol.317, No.1, (July 1998), pp. 91-94, ISSN0040-6031
- Martinez, D.; Tames, R.S. & Revilla, M.A. (1999). Cryopreservation of in vitro-grown shoot-tips of hop (*Humulus lupulus* L.) using encapsulation/dehydration. *Plant Cell Reports*, Vol.19, No.1, (November 1999), pp. 59-63, ISSN0721-7714
- Matsumoto, T. & Sakai, A. (1995). An Approach to Enhance Dehydration Tolerance of Alginate-Coated Dried Meristems Cooled to -196-Degrees-C. *Cryo-Letters*, Vol.16, No.5, (September 1995), pp. 299-306, ISSN0143-2044
- Murashige, T. & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum*, Vol.15, No.3, (1962), pp. 473-&, ISSN0031-9317
- Niino, T. & Sakai, A. (1992). Cryopreservation of Alginate-Coated Invitro-Grown Shoot Tips of Apple, Pear and Mulberry. *Plant Science*, Vol.87, No.2, (1992), pp. 199-206, ISSN0168-9452
- Niino, T.; Sakai, A.; Yakuwa, H. & Nojiri, K. (1992). Cryopreservation of In vitro-Grown Shoot Tips of Apple and Pear by Vitrification. *Plant Cell Tissue and Organ Culture*, Vol.28, No.3, (March 1992), pp. 261-266, ISSN0167-6857
- Nishizawa, S.; Sakai, A.; Amano, Y. & Matsuzawa, T. (1993). Cryopreservation of Asparagus (*Asparagus-Officinalis* L) Embryogenic Suspension Cells and Subsequent Plant-Regeneration by Vitrification. *Plant Science*, Vol.91, No.1, (1993), pp. 67-73, ISSN0168-9452
- Paulet, F.; Engelmann, F. & Glaszmann, J.C. (1993). Cryopreservation of Apices of In-Vitro Plantlets of Sugarcane (*Saccharum* Sp Hybrids) Using Encapsulation Dehydration. *Plant Cell Reports*, Vol.12, No.9, (July 1993), pp. 525-529, ISSN0721-7714
- Reed, B.M.; Schumacher, L.; Wang, N.; D'Achino, J. & Barker, R.E. (2006). Cryopreservation of bermudagrass germplasm by encapsulation dehydration. *Crop Science*, Vol.46, No.1, (January 2006), pp. 6-11, ISSN0011-183X
- Sakai, A. & Engelmann, F. (2007). Vitrification, encapsulation-vitrification and droplet-vitrification: A review. *Cryoletters*, Vol.28, No.3, (May 2007), pp. 151-172, ISSN0143-2044
- Sakai, A.; Kobayashi, S. & Oiyama, I. (1991). Survival by Vitrification of Nucellar Cells of Navel Orange (*Citrus-Sinensis* Var *Brasiliensis* Tanaka) Cooled to 196-Degrees-C. *Journal of Plant Physiology*, Vol.137, No.4, (February 1991a), pp. 465-470, ISSN0176-1617

- Sarkar, D. & Naik, P.S. (1998). Cryopreservation of shoot tips of tetraploid potato (*Solanum tuberosum* L.) clones by vitrification. *Annals of Botany*, Vol.82, No.4, (October 1998), pp. 455-461, ISSN0305-7364
- Schafer-Menuhr, A.; Muller, E. & Mix-Wagner, G. (1996). Cryopreservation: an alternative for the long-term storage of old potato varieties. *Potato Research*, Vol.39, No.4, (1996), pp. 507-513, ISSN0014-3065
- Sestak, J. & Zamecnik, J. (2007). Can clustering of liquid water and thermal analysis be of assistance for better understanding of biological germplasm exposed to ultra-low temperatures. *Journal of Thermal Analysis and Calorimetry*, Vol.88, No.2, (May 2007), pp. 411-416, ISSN1388-6150
- Seufferheld, M.J.; Stushnoff, C.; Forsline, P.L. & Gonzalez, G.H.T. (1999). Cryopreservation of cold-tender apple germplasm. *Journal of the American Society for Horticultural Science*, Vol.124, No.6, (November 1999), pp. 612-618, ISSN0003-1062
- Suzuki, M.; Tandon, P.; Ishikawa, M. & Toyomasu, T. (2008). Development of a new vitrification solution, VSL, and its application to the cryopreservation of gentian axillary buds. *Plant Biotechnology Reports*, Vol.2, No.2, (June 2008), pp. 123-131, ISSN1863-5466
- Tanaka, D.; Niino, T.; Isuzugawa, K.; Hikage, T. & Uemura, M. (2004). Cryopreservation of shoot apices of in-vitro grown gentian plants: Comparison of vitrification and encapsulation-vitrification protocols. *Cryoletters*, Vol.25, No.3, (May 2004), pp. 167-176, ISSN0143-2044
- Towill, L.E. (1990). Cryopreservation of Isolated Mint Shoot Tips by Vitrification. *Plant Cell Reports*, Vol.9, No.4, (August 1990), pp. 178-180, ISSN0721-7714
- Tyler, N.; Stushnoff, C. & Gusta, L.V. (1988). Freezing of Water in Dormant Vegetative Apple Buds in Relation to Cryopreservation. *Plant Physiology*, Vol.87, No.1, (May 1988), pp. 201-205, ISSN0032-0889
- Uragami, A.; Sakai, A.; Nagai, M. & Takahashi, T. (1989). Survival of Cultured-Cells and Somatic Embryos of *Asparagus-Officinalis* Cryopreserved by Vitrification. *Plant Cell Reports*, Vol.8, No.7, (October 1989), pp. 418-421, ISSN0721-7714
- Wu, Y.J.; Engelmann, F.; Zhao, Y.H.; Zhou, M.D. & Chen, S.Y. (1999). Cryopreservation of apple shoot tips: Importance of cryopreservation technique and of conditioning of donor plants. *Cryo-Letters*, Vol.20, No.2, (March 1999), pp. 121-130, ISSN0143-2044
- Wu, Y.J.; Zhao, Y.H.; Engelmann, F.; Zhou, M.D.; Zhang, D.M. & Chen, S.Y. (2001). Cryopreservation of apple dormant buds and shoot tips. *Cryoletters*, Vol.22, No.6, (November 2001), pp. 375-380, ISSN0143-2044
- Zámečník, J. & Faltus, M. (2009). Evaluation of Thermograms from Differential Scanning Calorimeter. *Cryoletters*, Vol.30, No.5, (September 2009), pp. 388-388, ISSN0143-2044
- Zámečník, J, Faltus, M. & Bilavčík, A. (2007). Cryoprotocols used for cryopreservation of vegetatively propagated plants in the Czech cryobank. *Advances in Horticultural Science*, Vol.21, (2007), pp. 247-250,

- Zamecnik, J.; Faltus, M.; Kotkova, R. & Hejnak, V. (2011). Glass transition determination in *Allium* shoot tips after dehydration. *Acta Hort. (ISHS)* 908 (April 2009), pp. 33-38, ISSN0567-7572
- Zhao, Y.H.; Wu, Y.J.; Engelmann, F. & Zhou, M.D. (2001). Cryopreservation of axillary buds of grape (*Vitis vinifera*) in vitro plantlets. *Cryoletters*, Vol.22, No.5, (September 2001), pp. 321-328, ISSN0143-2044
- Zhao, Y.H.; Wu, Y.J.; Engelmann, F.; Zhou, M.D.; Zhang, D.M. & Chen, S.Y. (1999). Cryopreservation of apple shoot tips by encapsulation-dehydration: Effect of preculture, dehydration and freezing procedure on shoot regeneration. *Cryo-Letters*, Vol.20, No.2, (March 1999), pp. 103-108, ISSN0143-2044

IntechOpen



## **Current Frontiers in Cryopreservation**

Edited by Prof. Igor Katkov

ISBN 978-953-51-0302-8

Hard cover, 462 pages

**Publisher** InTech

**Published online** 14, March, 2012

**Published in print edition** March, 2012

Almost a decade has passed since the last textbook on the science of cryobiology, *Life in the Frozen State*, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Jiří Zámečník, Miloš Faltus, Alois Bilavčík and Renata Kotková (2012). Comparison of Cryopreservation Methods of Vegetatively Propagated Crops Based on Thermal Analysis, *Current Frontiers in Cryopreservation*, Prof. Igor Katkov (Ed.), ISBN: 978-953-51-0302-8, InTech, Available from:  
<http://www.intechopen.com/books/current-frontiers-in-cryopreservation/comparison-of-cryopreservation-methods-of-vegetatively-propagated-crops-based-on-thermal-analysis>

**INTECH**  
open science | open minds

### **InTech Europe**

University Campus STeP Ri  
Slavka Krautzeka 83/A  
51000 Rijeka, Croatia  
Phone: +385 (51) 770 447  
Fax: +385 (51) 686 166  
[www.intechopen.com](http://www.intechopen.com)

### **InTech China**

Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821



© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](https://creativecommons.org/licenses/by/3.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen