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# Analysis of freezable water content by DSC for apple dormant bud cryopreservation

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**Abstract**: Dormant bud cryopreservation is a valuable tool for the germplasm of temperate trees preservation. Freezable water content, as an important cryopreservation characteristic, was analysed by DSC. Scions of modern apple tree varieties (*Malus domestica*) were collected during the winter season from orchards, freeze-dehydrated to 26–32% of water content at –5°C and cryopreserved by the two-step procedure. Samples were rehydrated in moist peat after 4 months of cryopreservation and grafted on rootstocks in a field. Regeneration by sprouting buds on rootstocks was evaluated. The varieties were divided into three groups according to their water content and percentage of crystallized water. The regeneration in the three groups decreased with decreasing bud water content. Regeneration was higher than 80% in most of the varieties and reached 100% in almost 1/3 of the tested varieties. In only one variety, was regeneration lower than 40%, which is still a high regeneration rate for considering apple genotypes as successfully cryopreserved. According to this study, the two-step cryoprotocol was successfully optimized for Central European conditions.

**Keywords**: apple tree; vegetatively propagated species; winter dormancy; conservation, cryopreservation techniques; differential scanning calorimetry

Apple tree (*Malus domestica*) belongs to the major temperate fruit crops (Dirlewanger et al. 2004). That is why breeding is processed intensively and there exist many genebanks of culture genotypes and important parental genotypes. *In situ* genebanks of plants grown in orchards are endangered by abiotic and biotic factors, especially by fireblight, a common disease of *Rosaceae* caused by *Erwinia amylovora,* widespread in the Central Europe. *In situ* plants as well as *in vitro* culture collections are based on growing organisms in which metabolic and other life processes occur and make mutations and genotype genome changes during their lives possible. Cryopreservation of apple dormant buds has started to be used for germplasm long-term storage as an alternative to standard methods for genetic resources conservation. Furthermore, it was proved that no genetic changes occur during cryopreservation in apple *in vitro* cultures (Hao et al. 2001). During cryopreservation procedure, viable plant parts have to survive temperatures far below zero (Sakai 1965; Ružić et al. 2014). Temperate woody plants developed many mechanisms for surviving frosts, one of them is extracellular water freezing (water freezes outside the cells in the apoplast) which protects cells from damage by intracellularly growing ice crystals (Sakai 1965; Quamme et al. 1973; Towill, Bonnart 2005). These principles are used in current cryoprotocols since water freezes slowly outside the cells during the first cryo-

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protocol step (cooling to –30°C and keeping –30°C for 24 hours) and remaining liquid water does not harm cell structure lethally (Tyler et al. 1988). The intracellular contents become concentrated as a result of the previously mentioned freeze dehydration to an appropriate water content. The cells are protected by cell wall from growing ice crystals, which are located in extracellular spaces, and cell volume is able to convert directly to an amorphous solid state called biological glass during cooling into ultralow temperatures (immersion into liquid nitrogen). This glassy state stops all metabolic or biochemical processes in cells or plant tissues, and allows the biological matter to survive ultralow temperatures, which are used for conservation in liquid nitrogen. To evaluate thermal events occurring in the sample during the cryopreservation processes the Differential scanning calorimetry (DSC) is used. The DSC is a physical analytical method to assess thermal events regarding heat flow, which occur in the sample during cooling and warming. This method is able to detect glass transition temperature of so-called biological glass that is a glassy state of cell solution content.

Many efficient plant cryoprotocols were developed in the USA at the USDA-ARS National Center for Genetic Resources Cryopreservation in Fort Collins, (Forsline et al. 1998b; Seufferheld et al. 2006; Towill, Bonnart 2005) and a great amount of genotypes was cryopreserved (Towill et al. 2004). Further investigation in mechanisms and physiological aspects of cryopreservation is continuing in Europe e.g. Denmark (Toldam-Andersen et al. 2007; Vogiatzi et al. 2010; Bilavcik et al. 2015), Germany (Höfer 2007), where some cultural apple varieties and landraces were successfully cryopreserved following the protocols developed at the USDA-ARS, USA. In the Czech Republic, apple dormant buds have not been cryopreserved until now, although this method is very worthwhile for the long-time protection of old regional varieties, which are grown in orchards in the Czech Republic. They often hold genetic predispositions for resistance to diseases, e.g. *Venturia inaquaelis* (Boček 2008). These varieties are often located in a few plots in the countryside and so their existence is endangered. The aim of this work was to evaluate the significance of the quantity of frozen water content determined by thermal analysis in a two-step protocol for cryopreservation of apple varieties in the climatic conditions of Central Europe.

## MATERIAL AND METHODS

One year old scions of 31 apple varieties (Table 1) were collected from commercial mother plantations, SEMPRA Litoměřice s.r.o., or from the orchard of Crop Research Institute (CRI) in Prague, in January and February (2010). The scions collected from SEMPRA Litoměřice s.r.o. were transferred immediately to CRI. In the CRI, scions were wrapped in plastic bags and stored in a freezing box for 3 days at –3°C. After that, the scions were cut into 180–200 uninodal segments, 35 mm long, with vegetative buds in the middle of them. Segments were put into open-mesh-bags and let to freeze dehydrate in freezing box at –2°C ± 1°C down to 26–32% of water content on a fresh weight basis (f.w.b.). Dry silica gel was put into the freezing box below the bags with dormant buds twice a week for faster dehydration. The bags were weighed weekly and later daily to control water losses. The estimated water content of individual varieties was calculated from the weighing values during the dehydration process. The fresh weight of segments before the beginning of dehydration, just after cutting, and second weighing of the same segments after their drying to constant weight, in a dryer at 85°C were measured. The water content was calculated as a percentage part of water from the fresh sample. After reaching the desired dehydration level, the freeze-dehydrated segments were transferred from bags to plastic tubes (20–25 segments in one tube, 7 tubes per variety) and put into a programmable controlled freezer to process the first step of cryoprotocol – cooling from –5°C to –30°C by –1°C/h and kept at –30°C for 24 hours. Subsequently, tubes in paper boxes were transferred from the freezer and put in liquid nitrogen (LN) vapours (approximately –135°C) for 45 minutes. Then, the paper boxes with tubes were quickly transferred into LN at –196°C. The final water content of cryopreserved bud segments was measured by weighing 5 randomly sampled segments simultaneously with starting of the freezing.

Samples of all varieties, one tube per variety, were removed from LN after 4 months and put in a cold chamber at 2°C ± 1°C in dark overnight to thaw slowly, then put in plastic bags with moist white peat to rehydrate in the cold chamber at 2°C ± 1°C for 14 days. After rehydration, buds from the segments were grafted by chip budding on apple rootstocks MM106 grown in the field, 2 buds per rootstock. Grafting and taking care of plants was

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Table 1. Regeneration of 31 apple varieties (three varieties repeated) after cryopreservation by two step protocol

| Variety | No. of dehydration days | Water on a f.w.b. in bud segments (%) | Regenerated buds (%) |
|---|---|---|---|
| **Group 1** | | | |
| 'Selena' | 12 | 32.1 | 83 |
| 'Mc Intosh' | 18 | 31.9 | 84 |
| 'Prima' | 15 | 31.5 | 65 |
| 'Gloster' | 15 | 31.1 | 100 |
| 'Jonagold' | 22 | 31.0 | 100 |
| 'Idared' | 39 | 31.0 | 100 |
| 'Sampion Red' | 15 | 31.0 | 89 |
| 'Rubinstep' | 20 | 30.1 | 100 |
| 'Melrose'* | 32 | 30.0 | 91 |
| 'Meteor' | 20 | 30.0 | 96 |
| Average ± SD | 21 ± 8.0 | 31.0 ± 0.70 | 91a ± 10.7 |
| **Group 2** | | | |
| 'Rajka' | 20 | 29.9 | 87 |
| 'Dione' | 22 | 29.8 | 100 |
| 'Melodie' | 15 | 29.6 | 100 |
| 'Melrose' | 22 | 29.6 | 100 |
| 'Topaz' | 21 | 29.5 | 42 |
| 'Chodske' | 15 | 29.2 | 100 |
| 'Rubin'* | 32 | 29.2 | 91 |
| 'Hana' | 15 | 29.2 | 36 |
| 'Sampion' | 21 | 29.1 | 84 |
| 'Denar'* | 33 | 29.0 | 91 |
| 'Daria' | 21 | 28.9 | 96 |
| 'Dezert' | 34 | 28.9 | 88 |
| 'Golden Del.' | 28 | 28.8 | 96 |
| 'Rubin' | 21 | 28.6 | 82 |
| 'Sampion'* | 33 | 28.6 | 100 |
| 'Angold' | 22 | 28.0 | 100 |
| Average ± SD | 23 ± 6.3 | 29.1 ± 0.49 | 87a ± 19.2 |
| **Group 3** | | | |
| 'Domino' | 34 | 27.9 | 100 |
| 'Dark Rubin' | 20 | 27.7 | 65 |
| 'Rubinola' | 20 | 27.6 | 85 |
| 'Julie' | 22 | 27.2 | 75 |
| 'Jarka' | 22 | 26.4 | 88 |
| 'Zvonkove' | 34 | 26.4 | 92 |
| 'Goldstar' | 21 | 26.1 | 48 |
| 'Prusvitne' | 25 | 26.1 | 96 |
| Average ± SD | 25 ± 5.5 | 26.9 ± 0.70 | 81a ± 16.4 |

means not sharing the same letter are significantly different at the 0.05 level of probability; *varieties from Crop Research Institute's orchards; f.w.b. – fresh weight basis

ensured by SEMPRA Litoměřice s.r.o., a professional fruit-tree grower, in their orchard. Sprouting of buds was evaluated 7 weeks after grafting.

Differential scanning calorimetry (Dual Sample Cell DSC TA 2920) was performed in buds prepared for cryopreservation and stored in plastic tubes in freezing box at –2°C to assess thermal characteristic as content of crystallised water and onset of ice nucleation temperature. A set of selected cryopreserved varieties at various stages of dehydration was measured. The buds were dissected from dehydrated segments and closed in hermetic aluminium pans, which were cooled from –5°C to –120°C and then warmed up to 20°C at the cooling/heating rate of 10°C/min. The heat flow of the samples was measured during this process. Then, samples were dried out at 105°C to ascertain their water content for comparison with the content of crystallised water. The results were analysed with TA Instruments Universal Analysis 2000 software. Microsoft Excel 2016 and Statistica 13.3. were used for statistical analysis.

## RESULTS AND DISCUSSION

Water content of bud segments decreased from 32.1 to 26.1% on a f.w.b. during their dehydration (Table 1). Dehydration lasted from 12 days (e.g. 'Selena') to maximally 39 days ('Idared'). According to Forsline et al. (1998b), the dehydration period of bud segments to 30 ± 2% varies among varieties, and depends on the size of scion diameter. The thinner scions dehydrate faster than thicker ones. Forsline et al. (1998b) experienced dehydration periods in various varieties from 4 to 6 weeks.Volk et al. (2009) reached the water content of 30% on a f.w.b. in *Faxinus* dormant buds of similar size in 4–7 days. Their faster dehydration might be affected by previous up to a 5 month-long term of storage of the ash budwood at –5°C before sample preparation. Not only the effect of different plant species, but also the effect of the pre-storage conditions (temperature and duration) of stock material must be taken in consideration for setting the dehydration period of the bud segments. The varieties were divided into three groups according to the level of dehydration. The first group ranged from the 30.0 to 32.1%, the second group ranged from 28.0 to 29.9 and the third group from 26.1 to 27.9% of water content on a f.w.b.

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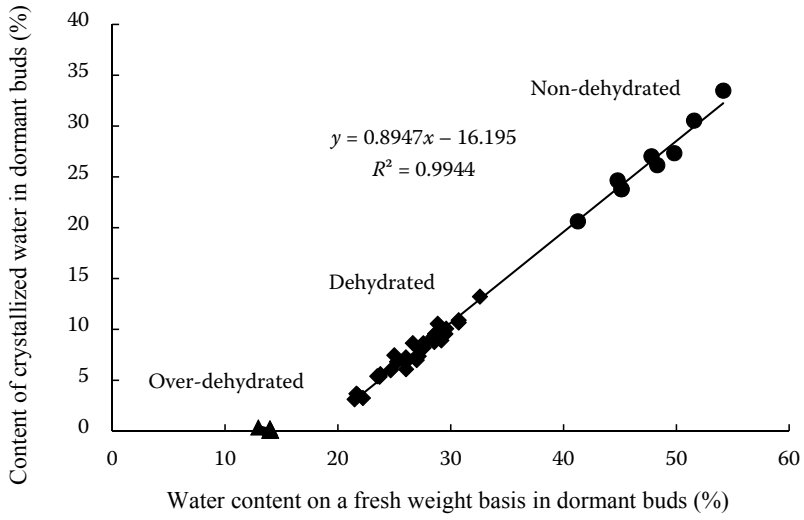


Fig. 1. Dependence of content of crystallized water in dormant apple buds of different varieties on total water content on a fresh basis in dormant buds

The DSC thermograms were evaluated during heating of buds of different dehydration level (Figs 1 and 2). Heat flow was measured as the amount of heat energy necessary to be spent to warm up the sample by constant warming rate 10°C/min. The state changes (from crystal to liquid state) are expressed in form of inverse peaks since more heat energy is spent for constant warming up the sample during ice melting. The more crystallized water in the sample, the more heat was needed for the state change. (Fig. 3c – fresh bud – 50% of water on a f.w.b., Fig. 3b – bud dehydrated to 30% water on a f.w.b.). Over-dehydrated samples with minimum crystallized water showed almost no peak – Fig. 3a. These diagrams enable an accurate assessment of the content of crystallized water and onset temperature by specification of the peak area. Differential scanning calorimetry analysis made in buds at various dehydration levels showed, that independently of the variety, the content of crystallized water correlated linearly with the total content of water in buds assessed by weighing of samples before and after freeze-drying procedure (Fig. 1). The correlation between water content and content of crystallized water (Fig. 1) was highly statistically significant ($P < 0.01$) and showed that the bud segments could withstand crystallisation of 8.9–12.4% of total water on a f.w.b. in their bud tissues. This level of crystallization was reached when the buds were freeze-dehydrated before cryopreservation to 29.1 ± 0.49% water content on a f.w.b. Insignificant content of crystallized water in tissues was detected, when the water content in bud decreased under 18.1% on a f.w.b. The varieties were divided into

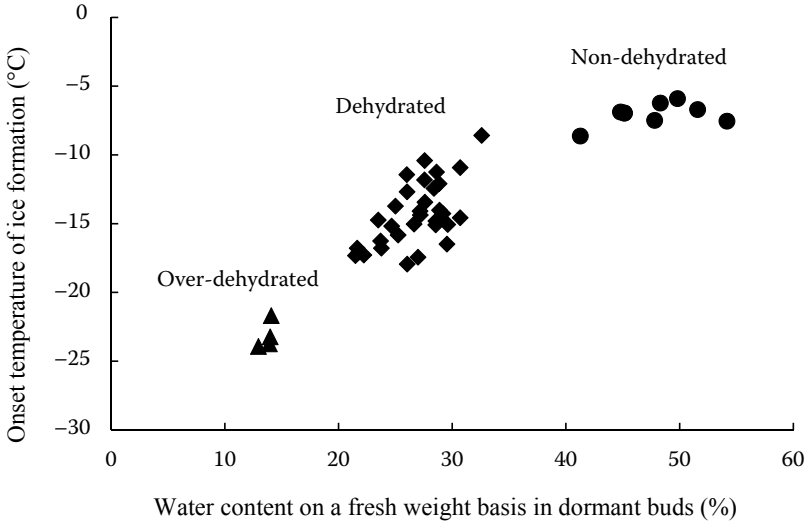


Fig. 2. Onset temperatures of ice formation in dependence on total water content on a fresh basis in dormant buds

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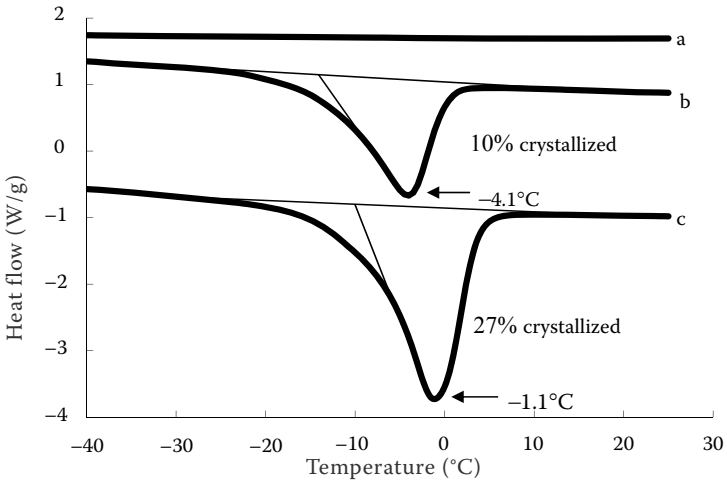


Fig. 3. DSC heating thermogram of dormant apple buds of different dehydration levels. From an over-dehydrated bud below 18.1% of water content on fwb (a), a bud dehydrated to the level of 30% (b), and a freshly collected non-dehydrated bud of 50% water content (c)

three categories according to their final water content after dehydration (Figs 1 and 2). The first category represented non-dehydrated samples where the water content in buds was between 41% and 55% on a fwb. The second category represented samples at middle level of dehydration (between 21% and 33%) which still allowed the remaining water to form ice crystals. These two categories showed the linear dependence of content of crystallized water on the total water content found in tissue. The third category represented varieties dehydrated below the limit for the water crystallisation and therefore these samples were excluded from the correlation. Bud water content of four measured varieties was under the limit 18.1% on a f.w.b. (reached 13%–14%) and the percentage of crystallized water was close to zero (0.4% water content on a f.w.b.). The analysis showed that the content of 'non-freezable' water was very close to 18.1% on a f.w.b. in all tested apple varieties, and all remaining water above this level crystallized. Whereas some other studies investigated percentage of freezable water in dormant buds in critical cryopreservation steps (Tyler et al. 1988; Vogiatzi et al. 2012), this study focused on total contents of frozen and unfreezable water in buds and various varieties. The lower water content of buds, the lower the onset temperature of ice formation temperatures was detected. The onset temperatures of non-dehydrated samples did not exceeded –9°C, whereas the onset temperatures of dehydrated samples at levels of 21%–33% water content on a fwb ranged from –9% to –18°C and of samples over-dehydrated to 13–14% ranged from –21 to –24°C.

The buds sprouted into shoots and regeneration rates and qualities of the tested varieties were observed seven weeks after grafting on rootstocks. The cooperation with a commercial nursery was important because high quality rootstock for cryopreserved buds was provided and experienced workers doing the chip budding after cryopreservation were crucial. Inexperienced grafting can negatively affect the regeneration results markedly. Most of sprouted shoots formed standard newly sprouted twigs (Fig. 4a). In some cases, it was apparent that the main meristem in the bud died out and secondary buds started to develop shoots with the delay (Fig. 4b). They appeared randomly within some varieties in an amount of 1 or, maximally 2 buds per variety. In contrary, Vogiatzi et al. (2011) mention that too big buds of apple varieties tend to have problems with regeneration after cryopreservation, and the presence of secondary buds significantly affect subsequent regeneration in such cases. A small amount of buds, from 1 to 2 per variety, in varieties 'Zvonkove', 'Sampion', 'Rubin', 'Denar', 'Jonagold' developed flowers (Fig. 4c). Regardless of that, these buds were able to develop leaves and sprouts later, and so were considered as regenerating. The second observation of regenerating plants was performed 15 weeks after grafting to assess the final regeneration rates. Regeneration was higher than 80% in most of the varieties and reached 100% in 10 cases (Table 1). Only one variety had a regeneration rate lower than 40%, which was the minimal amount for considering an apple genotype as successfully cryopreserved according to Towill et al. (2004). The results markedly outweighed results of some European researchers from previous years, such as experiments in Poland with Polish varieties, 2007–2008 (Wolińsky – personal communication) or in Germany where the same varieties as in our study were investigated too

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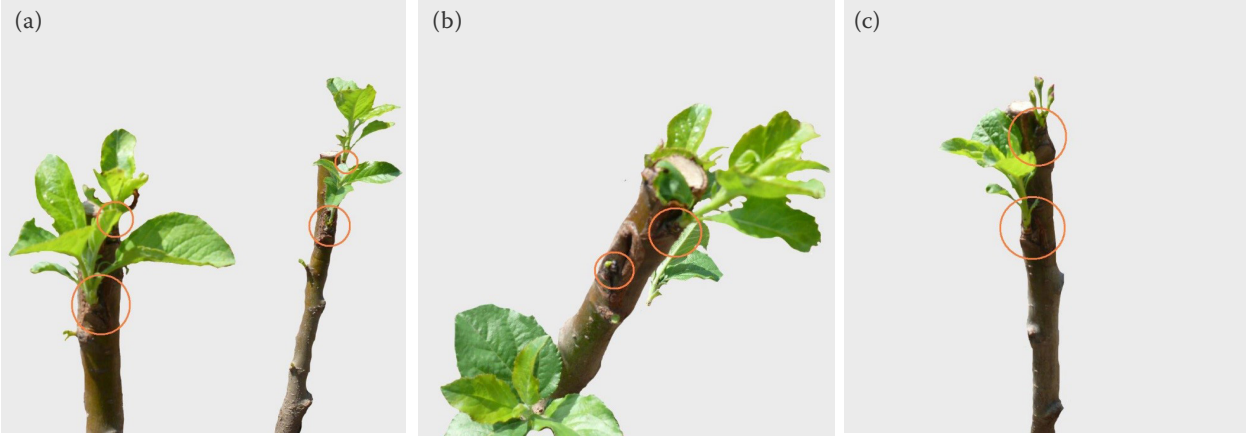


Fig. 4. Sprouting buds of apple varieties after cryopreservation. (a) Normally developed shoots from primary buds ('Jonagold '), (b) sprouting secondary buds, primary buds not alive ('Jonagold'), and (c) a sprouted flower bud before blooming ('Rubin').

(Hofer 2007). Volk et al. (2009) obtained similar results of survival of cryopreserved dormant buds of different *Fraxinus* species.

Although water content and freezable water content can influence the success of cryopreservation (Tyler et al. 1988), the assessed regeneration rates were neither dependent on actual water content in dehydrated bud segments within observed dehydration scale, nor even on length of dehydration period (although these factors could probably affect the results in some our cases, e.g. in 'Goldstar'). Water content of samples, measured for controlling dehydration process at time of starting cryoprotocols, ranged from 26.1% ('Prusvitne') to 32.1% ('Selena'); eight varieties were dehydrated in the range of 26% to 28%. However, these higher dehydrations of recommended 30 ± 2% of water content obviously did not affect negatively the regeneration after cryopreservation. Only in one case ('Goldstar', 26.14% of water content), the regeneration was less than 50%, in other cases it reached almost 100% and was at least 82.8% ('Selena'). On the other hand, two varieties, dehydrated at the desired level, regenerated under the limit for successful cryopreservation or close to this limit: 'Topaz' (29.5% water content, 41.7% regeneration), 'Hana' (29.1% water content, 36.4% regeneration). Despite these variations, the method of initial water content estimation and further determination of water content levels based on regularly weighed samples can be recommended as a functional method of controlled dehydration.

Furthermore, cryopreservation success seemed to be unaffected by collecting the scions later than until half of January, as recommended (Towill et al. 2004; Bilavcik et al. 2015). Most of genotypes collected on January 21 and February 4 showed similarly good results such as genotypes collected on January 5 and January 7 – this was probably due to long-lasting winter in 2010. However, all three varieties, which showed insufficient regeneration, were collected on two later dates, which could influence their regeneration rates negatively in relation to their possibly higher sensitivity. In case of 'Sampion' collected on January 21 in mother orchard of Sempra Litoměřice showed regeneration of 84%, while the same variety collected on January 5 in orchard of CRI showed regeneration of 100% (Table 1). Physiological stages of various trees in various seasons seem to affect their regeneration abilities after cryopreservation too (Forsline et al. 1998b, Bilavcik et al. 2015). The suitable weather in winter 2010 could also play a role in regeneration rates. The temperatures were under 0°C for most days in winter and this could be crucial for successful cryopreservation. However, Jenderek et al. (2011) suggested that the temperature at the growing location may not hinder application of the dormant apple bud cryopreservation method to the extent previously speculated. Nevertheless, the effect of grafting season/conditions should be also taken into account. Volk et al. (2009) found even higher regeneration (35% vs. 79%) of the same control sample of *Fraxinus* grafted 18 month later (as opposed to 2 to 5 month). The reason of specificity of varieties in reaction on the cryoprotocol might be in the anatomical differences in the varieties, as well as their physiological responses on the stress factors during the cryopreservation procedure. Those stress factors induce production

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of reactive oxidative species the cryopreserved tissues must overcome and maintain the balance of the physiological environment in the cells (Prudente, Paiva 2017). Cryopreservation's success for greater number of species is dependent on the understanding of biochemical aspects of cryopreservation.

## CONCLUSION

The study represents a successful assessment of protocol for cryopreservation of apple tree varieties as genetic resources (Forsline et al. 1998b; Seufferheld et al. 2006), with modifications depending on distinct laboratory equipment. High regeneration rates after cryopreservation were obtained, from 36 to 100% with the average 87 ± 16.8%. The regeneration in the three groups of varieties, assorted by the final water content, had a decreasing trend with decreasing bud water content. It was proved by the DSC that contents of freezable water in dehydrated buds of apple varieties linearly correlated with total contents of water in the buds, independently on a variety. Finding that buds withstood cryopreservation without damage with a recommended dehydration level of 26%–32% water content improves cryoprotocol utilization possibilities. Rare lower tolerances of particular varieties to applied cryoprotocol should have other causes than water content only. The protocol is a useful tool for cryopreservation of other desired apple varieties including old varieties and landraces. The cryoprotocol used enabled establishing a functional cryobank that serves as a backup of *in vitro* and *in situ* collections and also aids in the long-term storage of important genetic resources (e. g. old and regional varieties) for future use by researchers and breeders.

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