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

4,800
Open access books available

122,000
International authors and editors

135M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS BOOK CITATION INDEX INDEXED

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

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Chapter

Methods of Thermal Analysis as a Tool to Develop Cryopreservation Protocols of Vegetatively Propagated Crops

Stacy D. Hammond Hammond, Miloš Faltus and Jiří Zámečník

Abstract

Cryopreservation is considered to be a reliable biotechnological tool for the long-term conservation of vegetatively propagated plant germplasm. The technique is based on freezing plant tissues at an ultralow temperature. However, high water content in plant tissue can result in injury during the cooling and thawing processes. Water behavior in the process of cryopreservation can be assessed by the use of thermal analysis method. This chapter demonstrates how the use of heat flux-type differential scanning calorimetry (DSC) thermal analysis methods such as standard DSC, temperature-modulated DSC (TMDSC), and quasi-isothermal temperature-modulated DSC (QITMDSC) can be used to assess the amount of freezable water and verify if the tissue being used has reached glass transition as well as analyzing the thermal events during cooling and freezing to reduce crystallization and damage by frost. Here, you can find a guide on how these thermal analysis methods can be applied, through concrete examples of each method and their use in the development of a more reliable and precise cryopreservation protocol for vegetatively propagated plant species.

Keywords: differential scanning calorimetry, freezable water, glass transition, heat flux DSC, ice crystallization, quasi-isothermal temperature-modulated differential scanning calorimetry, temperature-modulated differential scanning calorimetry

1. Introduction

Cryopreservation is emerging as one of the most promising techniques for the long-term conservation of plant germplasm; it is also considered as an alternative method to safeguard plant species preserved by conventional conservation practices such as in vitro techniques and ex situ methods, i.e., seed and field collections, including vegetatively propagated crop species.

This conservation technique is based on the freezing of tissues (e.g., organs and shoots tips) from in vitro plantlets or field collections at extremely low temperatures, with the aim of reducing metabolic activity while at the same time maintaining the vitality of the tissue. The main principle of the method is based on cooling plant tissues at an ultralow temperature, usually by the use of liquid nitrogen (LN) which has a constant temperature of −196°C. The frozen tissue after the desired

period in ultralow temperature after rewarming is able to regenerate to healthy plants. However, this method is not as straightforward as it seems; freezing plant tissue can result in intracellular ice nucleation and subsequent ice crystallization leading to cell damage during cooling and/or rewarming of the samples.

Low water content minimizes ice crystallization. Hence, the objective status for cryopreservation is to reduce the water content in the plant tissue. By this procedure it obtains a glassy state to avoid the formation of lethal intracellular ice crystals in order to obtain optimal recovery and regeneration after cryopreservation. This can be achieved by the induction of vitrification status which is a glass induction by dehydration, the addition of cryoprotectants, and a very fast decrease in temperature.

Methods based on dehydration include osmotic dehydration, air desiccation, and freeze dehydration [1–3]. The state of the water in the plant during cooling/warming cycles is shown in **Figure 1** with highlighted thermally colored and its possible detection and quantification by DSC techniques.

Various plant vitrification solutions (PVS) are used for osmotic dehydration; these are usually labeled with a number according to the specific mixture of basic cryoprotectants and their concentrations. The main PVS are Luyet [4], Fahy [5], Steponkus [6], PVS1 [7, 8], PVS2 [9], PVS3, PVS4, PVS5 [10], VS6 [11], PVSL [12], and VSL [9]. These are composed of different concentrations and combinations of four main cryoprotectants: dimethyl sulfoxide, sucrose, glycerol, and ethylene glycol.

Each cryopreservation protocol is species- and genotype-specific and needs to be optimized accordingly. Hence, the standardization of the protocol is necessary to ensure and facilitate an effective cryo-storage of the plant germplasm. To achieve this, it is necessary to determine the optimal water content during dehydration, freezing in LN and thawing to avoid ice crystallization. Additionally, assessing the amount of freezable water and verifying if the tissue being used has reached glass transition as well as analyzing the thermal events during cooling and freezing are key factors in order to develop and standardize a reliable protocol.

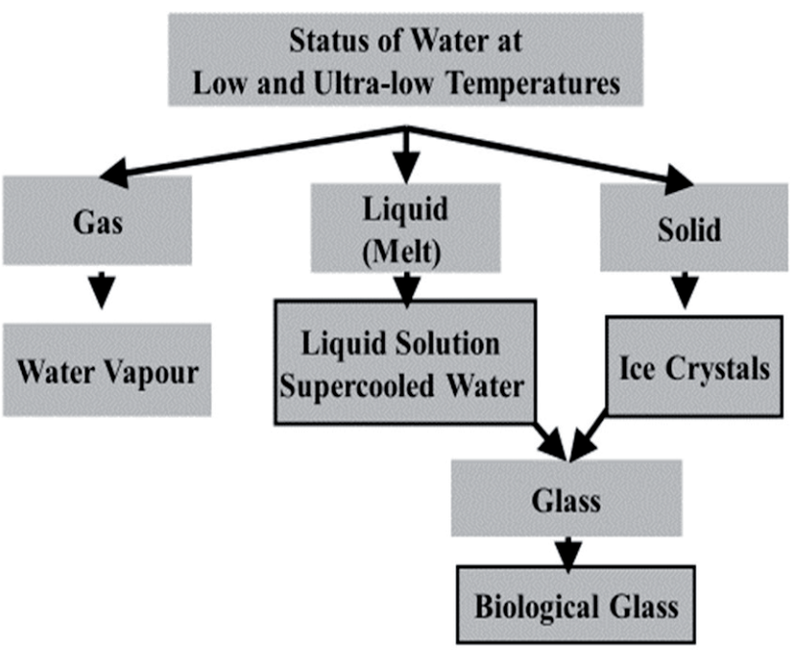

Figure 1.
State of water in biological objects for cryopreservation. Those highlighted in a black frame are possible to quantify by DSC.

This can be determined by the use of thermal analysis such as differential scanning calorimetry (DSC).

The use of DSC for checking the content of freezable water (or first-order water transitions freezing and melting) is applied for the cryopreservation of shoot tips [13–15], dormant buds/winter-hardy buds [16–18], pollen [19–21], and seeds [15, 22–24]. The development of cryopreservation protocols using DSC has been used for a number of plant species, i.e., potato [25], *Ullucus tuberosus* Cal. [26], *Oxalis tuberosa* Mol. [27], *Allium* species [19, 21, 28, 29], apple [30], and *Actinidia* spp. [31], among others. However, the method is still rarely used in cryobiology due to the lack of awareness of its use, application, and potential in this field. This chapter intends to broaden the knowledge and demonstrate the use of thermal analysis methods with heat flux-type DSC and their applicability in cryopreservation through a series of research carried out at the Crop Research Institute in Prague, Czech Republic. The results presented in this chapter represent original unpublished work archived at the authors' institution.

2. Differential scanning calorimetry

DSC is a thermal method that can be used to measure and determine the phase transitions for cryopreservation. Using a function of time and temperature, the main principle of this method is to measure the temperatures and heat flows associated with thermal transitions in plant material providing information on the endothermic or exothermic events or changes in heat capacity. This information can be used to determine the glass transition, the temperature of ice nucleation, melting, boiling, crystallization time, and kinetic reaction which are the most important characteristics useful for cryopreservation [16].

This method is based on applying a regulated decrease and increase in temperature while measuring the heat flow and temperature corresponding to the tested sample. The heat flux-type DSC 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°C min^{-1}. There are three different temperature modifications of the heat flux-type DSC: standard DSC gives a basic information about exothermic and endothermic signals and about glass transition presence, temperature-modulated DSC (TMDSC) separates kinetic and thermodynamic events in tested samples, and quasi-isothermal temperature-modulated DSC (QITMDSC) determines the exact measurement of heat capacity at equilibrium conditions. The principle, application, and use of each of these DSC modifications will be demonstrated within this chapter through a series of experiments carried out on vegetatively propagated species and cryoprotectants.

Thermal characteristics as melting point temperature, glass transition temperature, and proportion of freezable water content (percent of the fresh weight) were determined using a differential scanning calorimeters TA2920 (TA Instruments, USA) with refrigerated cooling system (RCS) in the temperature range from −60 to +20°C and Q2000 (TA Instruments, USA) with RCS or liquid nitrogen cooling system (LNCS) in the temperature range from −90 to +20°C or from −140 to +20°C, respectively. The cooling and heating rates were 10°C/min. or 1°C/min. For standard DSC or TMDSC, respectively. Temperature modulation was performed at 1°C amplitude of modulation and 60-second period. Aluminum, hermetically sealed pans were used for all samples to avoid water evaporation before and during measurement cycles. Sample size ranged from 5 to 20 mg. The purge gas was either nitrogen or helium.

3. Standard DSC measurement and result analyses

Standard DSC is a thermal analysis method used to carry out a total heat flow measurement of the sample in order to determine phase transitions such as crystallization, melting, and glass transition (**Figure 2**).

In terms of cryopreservation of plant tissue, this method of thermal analysis can help to improve the development of a more reliable protocol. It can be applied in the phase of optimization of the sample's preculture, acclimation, or dehydration to decrease the amount of freezable water content within the sample and in the risk of sample injury (**Figure 3**). The quality of cryo-sample preparation can be checked and modified to avoid the presence of cold crystallization in the sample during its thawing after cryo- storage for its recovery (**Figure 4**). This method can also be used to assess the T_g (glass transition temperature) and to determine safe storage temperatures for the samples.

3.1 Freezable water content determination

Freezable water content is probably the most important parameter which influences the injury of plant tissue during cryopreservation using cryoprotocol avoiding controlled freeze dehydration. The proper decrease in freezable water content is hence prerequisite for successful cryopreservation. Standard DSC method measures the endothermic and exothermic signal related to water freezing and thawing. Based on the knowledge of heat of fusion specific for water at 0°C (334 J/g/K) and total amount of energy used for the phase transition of water melting (ice crystals thawing), the total amount of frozen water can be calculated, and based on this finding, the optimal time of PVS3 treatment can be adjusted. Freezable water was detected in garlic shoot tips during PVS3 dehydration for a period up to 90 min (**Figure 5a**). After ice nucleation this water fraction can cause cell damage by ice crystal growth. Intracellular ice formation is in any case lethal for the plant cell. There was no freezable water in plants after 90 min of dehydration. The appropriate level of dehydration by PVS3 has to be

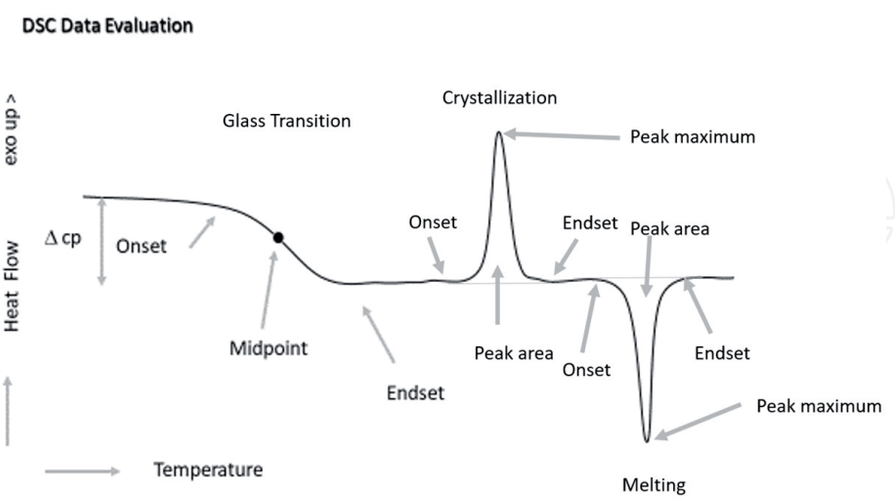

Figure 2.
Thermogram from differential scanning calorimeter during warming. The glass transition is characterized by the beginning of the heat flow change (onset), midpoint (in some cases inflection point), and finish of heat flow change (endset), characterized by change of heat capacity (ΔCp). Crystallization of supercooled water is an exothermic reaction, followed by the thawing of crystalized water. These events can be characterized by onset, midpoint, and endset temperatures and by event heat flow change. Based on analysis of the exothermic or endothermic events, the freezable water content can be calculated also in dehydrated samples. Additionally, this thermal analysis method is a powerful tool in the assessment of the sample's water behavior during cooling and warming; this includes supercooling, freezing, glass transition, cold crystallization, and melting which influence the sample's cryopreservation success.

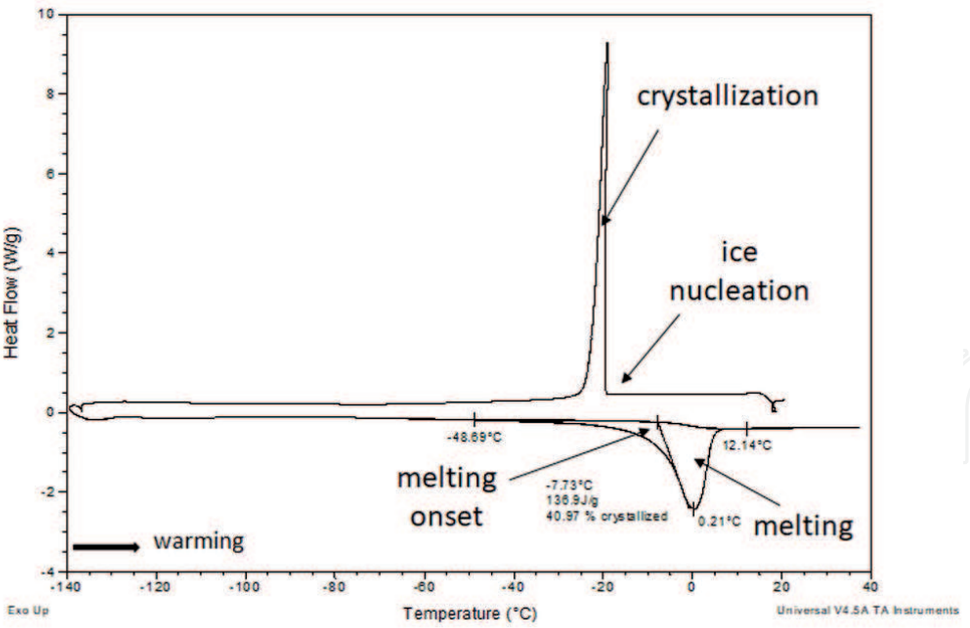

Figure 3.
Area of the melting peaks represents the heat of fusion needed for melting of the sample, related to the amount of frozen water in potato shoot tips (cv. "Arnika") after 1 h air dehydration (Q2000 + LNCA).

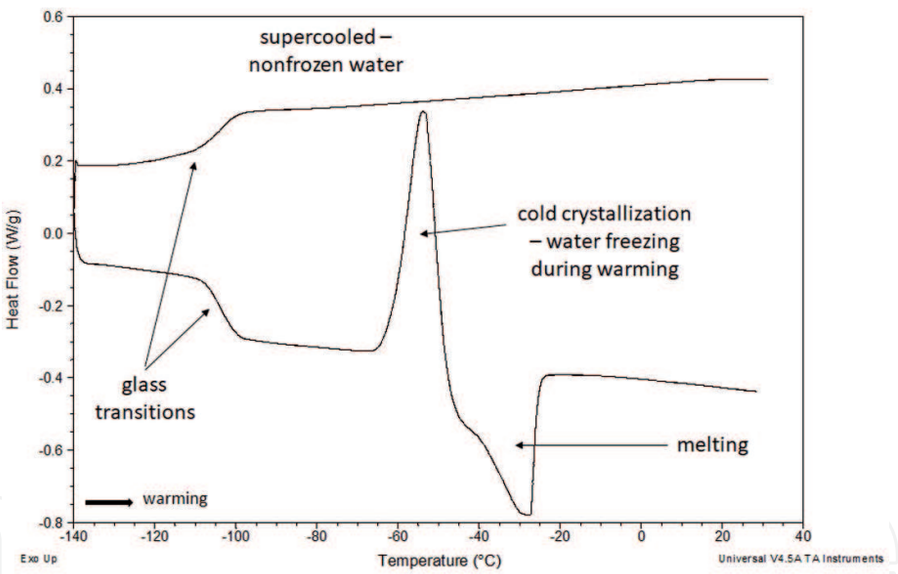

Figure 4.
Cold crystallization during the warming cycle in potato shoot tips (cv. "Arnika") in diluted PVS3 solution (Q2000 + LNCA).

controlled due to the decrease of survival and regeneration of shoot tips by excessive dehydration (**Figure 5b**). For successful cryopreservation with high regeneration rate, samples in narrow dehydration window of PVS3 must be used: with neglected or no freezable water (**Figure 5a**) and high regeneration rate due to dehydration (**Figure 5b**) after warming from ultralow temperatures.

3.2 Safe storage temperature determination

Safety and stability of plant sample stored at ultralow temperature are influenced by the temperature of glass transition. Throughout the period of cryopreservation, the sample has to be kept below the sample glass transition temperature.

In case of increase of storage above the sample glassy state temperature, the risk of sample damage by ice crystals occurs. Different cryopreservation methods resulted in different sample glass transition temperature. Thus, the selection of the cryopreservation method or cryoprotectant used can influence safe storage temperature. The most frequently used cryoprotectants for plant cryopreservation PVS2 and PVS3 are characterized by Tg of −114 and −90°C, respectively. When the 80%PVS3 solutions were used, the glass transition temperature was approximately −88°C in potato shoot tips dehydrated for 2 h (**Figure 6**).

Some cryoprotective solutions with a higher glass transition temperature can be used to increase the safe storage temperature and sample stability. Standard cryopreservation methods used in CRI is based on sucrose loading the shoot tips and subsequent air dehydration. This dehydration resulted in increased sucrose concentration and as a result increased in glass transition temperature. Thus, the glass transition temperature of potato shoot tips increased to approximately −23°C after 0.7 M sucrose loading and following air dehydration for 5 h (**Figure** 7).

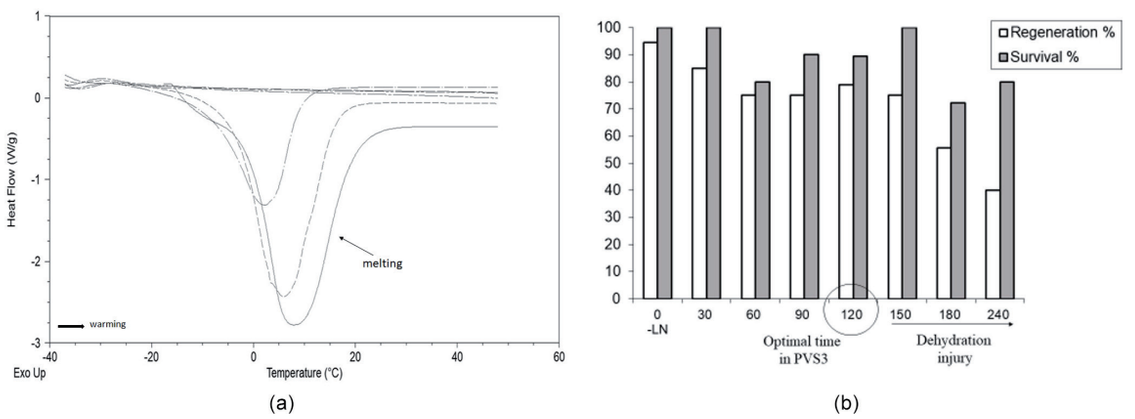

Figure 5.
(a) Frozen water content after different time of shoot tips of Allium sativum *"Djambul" in PVS3, after 30, 60, and 90 min (from largest to smallest peak). There is no detectable frozen water content in shoot tips for a longer time in PSV3 (TA 2920 + RCS). (b) Survival and regeneration after different time of shoot tips of* Allium sativum *"Djambul" in PVS3, after 30, 60, and 90 min (in this time interval, there was no regeneration after liquid nitrogen immersion, because of lethal injury from frozen water). The optimal time is 120 min in PVS3 because from this time the survival and regeneration rate decreased because of dehydration injury*

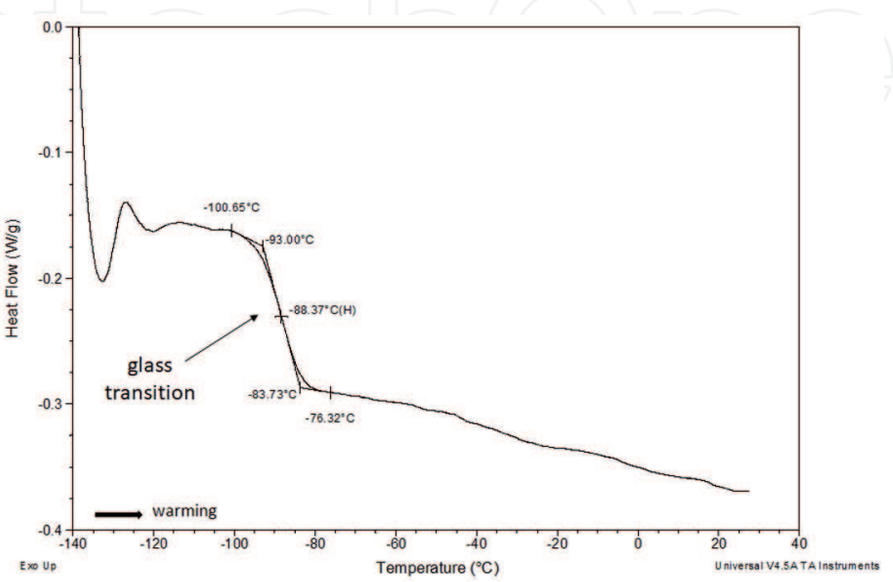

Figure 6.
Glass transition of potato shoot tips (cv. "Désirée") after 2 h dehydration in 80%PVS3 (Q2000 + LNCA).

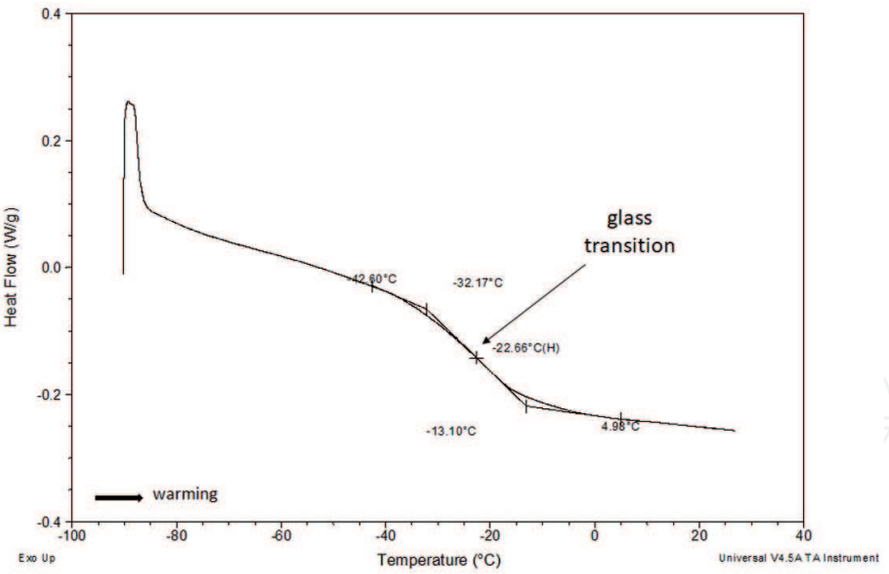

Figure 7.
Glass transition of potato shoot tips (cv. "Désirée") loaded in 2 M sucrose solution and subsequently dehydrated by air for 5 h (Q2000 + RCS).

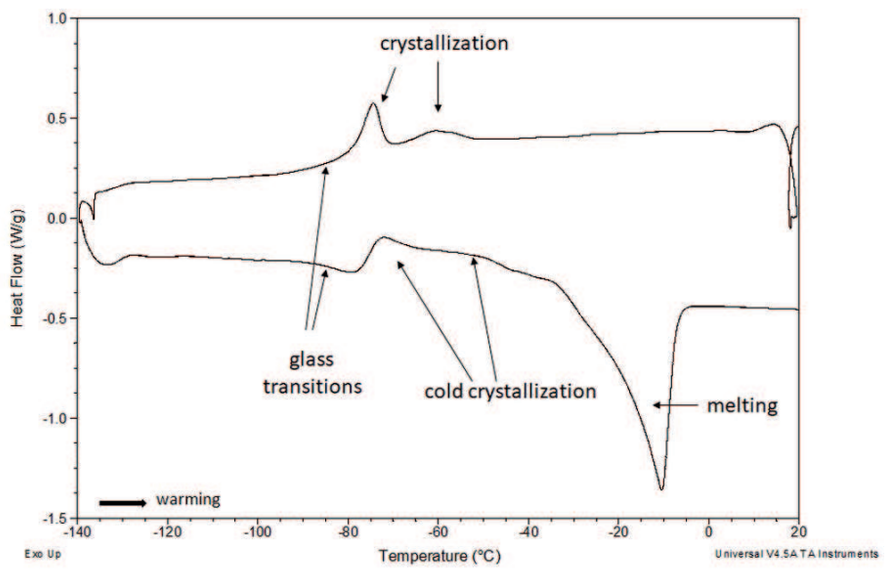

Figure 8.
Complex thermogram of 60% sucrose solutions including thermodynamic (glass transition, melting) and kinetic (crystallization) events (Q2000 + LNCA).

3.3 Benefits and limits of standard DSC measurements

Though this method of thermal analysis is easy and simple to use (most standard programs use simple methods with cooling/warming rates of 10°C min^{-1}) and provides a relatively fast real-time measurement of thermal characteristics in the course of dehydration of the sample (e.g., 30-min DSC program allows to perform measurements in every 30 min of dehydration), there are some limitations. One of these limitations is the measurement of the total heat flow signals which include both thermodynamic and kinetic signals; hence, the evaluation of thermodynamic signals may not be exact. Additionally, kinetic events like relaxation or crystallization make thermogram evaluation and event identification difficult and can mask the thermodynamic component of the total heat flow signal (**Figure 8**). The overlapping signals may not be identified. In such cases, there is the need to carry out a more detailed analysis of the specific thermal events by the use of TMDSC or QITMDSC.

4. Temperature-modulated DSC

TMDSC is the thermal analysis method (**Figure 9**) used to separate the total heat flow signal into thermodynamic and kinetic components. Hence, through this method, thermodynamic events such as melting, glass transition temperatures, and kinetic events such as crystallization and relaxations can be analyzed separately. Additionally, the freezable water content and the water behavior in the sample during cooling/warming cycles, like supercooling, freezing, glass transition, cold crystallization, and melting can be determined. This method also determines the Cp (specific heat capacity) and allows to specify its reversible Cp (thermodynamic) component.

This method of thermal analysis can help to improve the development of a cryopreservation protocol by improving the quality of the cryo-sample preparation. The method can identify kinetic events, which mask the thermodynamic events and which reveal unstable sample conditions. Due to the identification of the thermodynamic component of the heat flow signal by this method, the exact determination of freezable water content and the Tg in the sample is possible.

4.1 Separation of thermodynamic and kinetic events to identify the stability of plant material

This method of thermal analysis can help to improve the development of a cryopreservation protocol by improving the quality of the cryo-sample preparation. The method can identify kinetic events, which mask the thermodynamic events and which reveal unstable sample conditions. Due to the identification of the thermodynamic component of the heat flow signal by this method, the exact determination of freezable water content and the Tg in the sample is possible.

The total heat flow signal includes both thermodynamic and kinetic signals. That is why exact analysis in complex thermogram is difficult (**Figure 10**). Total heat flow showed some changes in the region from −61 to −12°C in 64% sucrose solution (from left to right): flat-shaped curve, high s-shaped curve, and peak. It is difficult to solve if the second event is a glass transition of melting. The TMDSC separated total heat flow into reversing and non-reversing signals. The only two

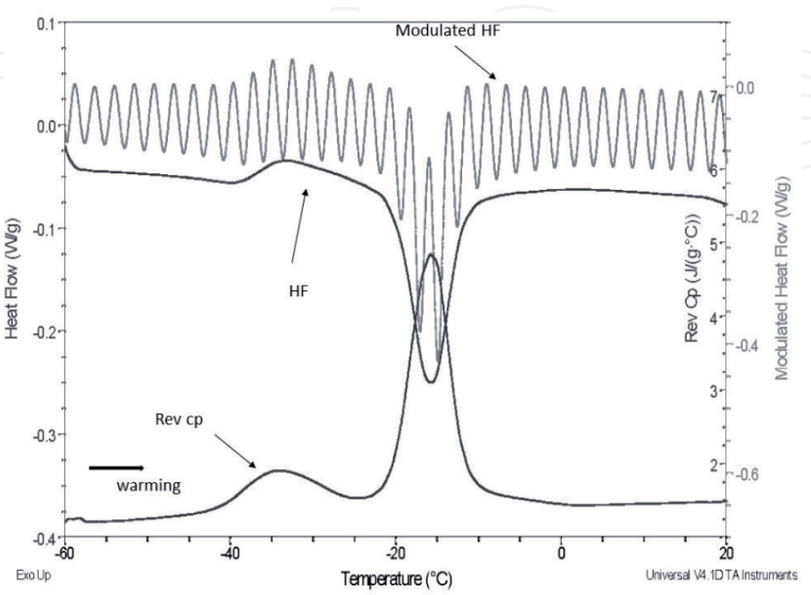

Figure 9.
The example of TMDSC result of thawing thermal event in Allium shoot tips. Note the reversing heat capacity (rev Cp) opposite to heat flux (TA 2920 + RCS).

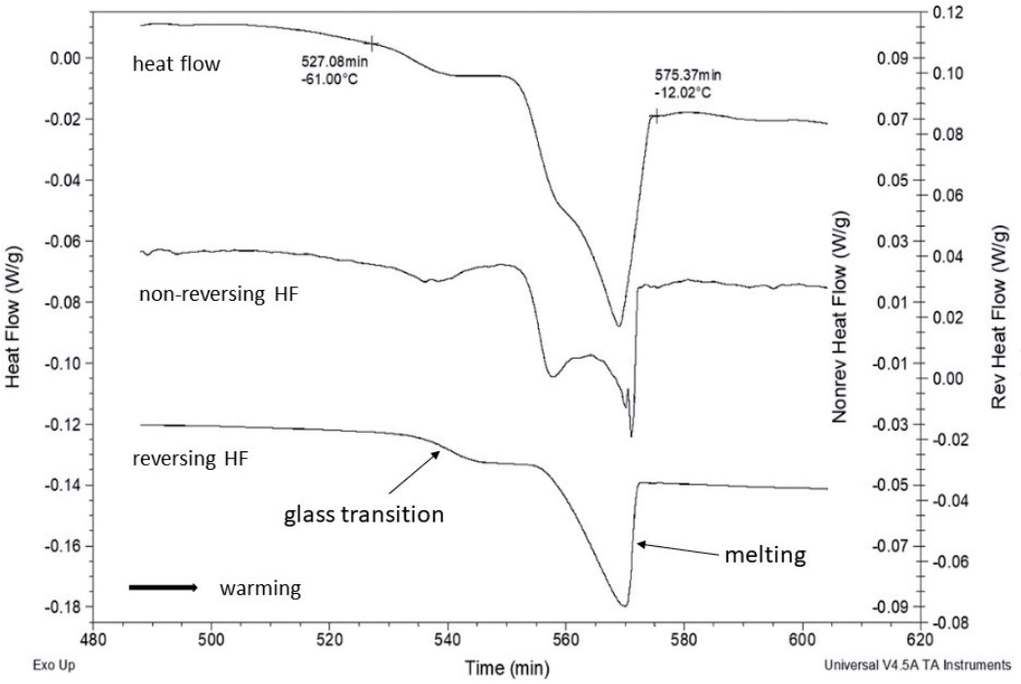

Figure 10.
Separation of total heat flow (HF) signal (upper) to non-reversing kinetic signal (middle) and reversing thermodynamic signal (bottom) in 64% sucrose solution (Q2000 + LNCA).

events were detected on reversing curve: glass transition and melting. The second event detected by standard DSC represents just kinetic event related to the crystallization of free water which is released before from glassy state during warming.

4.2 State of matter determination by a direct Cp measurement

The TMDSC can be also used for direct measurement of specific heat capacity (Cp). It changes with the change of the state of matter. The liquid state is characterized by a higher value of Cp than solid (crystal or ice) states. Phase changes, glass transition, and melting are characterized by a specific shape of Cp curve. The glassy state is characterized by an S-shaped curve of Cp baseline; on the other hand, melting is characterized by a peak (**Figure 11**).

Proper sample preparation for its successful cryopreservation is characterized by low-temperature glass transition curve but without melting peak presence. A decrease in the melting peak on the Cp curve is therefore necessary during sample preparation. An appropriate way of dehydration has to be applied to avoid plant tissue injury by excessive dehydration. Tolerance to dehydration is influenced by species, genotype, and by plant tissue or cells' physiological status. Pollen, besides orthodox seeds, is a plant material that can be dehydrated to very low water content without decrease of plant material vitality. Dehydrated pollen is characterized by the absence of a melting peak but the presence of glass transition s-shaped curve (**Figure 12**). Due to very low water content, the glass transition temperature is at high temperature (−18°C), which strongly increases the safe storage temperature of the material during cryopreservation.

4.3 Benefits and limits of TMDSC

Though if properly applied and analyzed, the method can be advantageous because of its exact measurement of thermodynamic events due to the separation of thermodynamic and kinetic signals and can also be useful in the identification of the overlapping events.

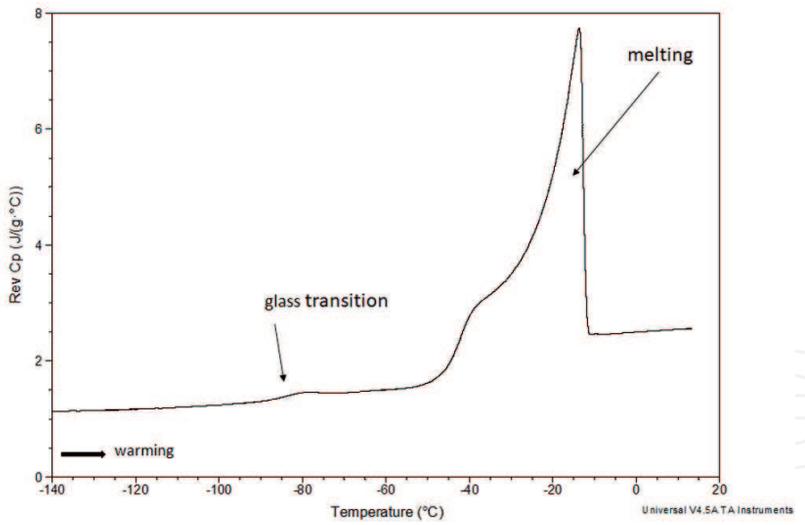

Figure 11.
Changes in reversible Cp during warming with detection of glass transition and melting in 20% sucrose solution by TM DSC (Q2000 + LNCA).

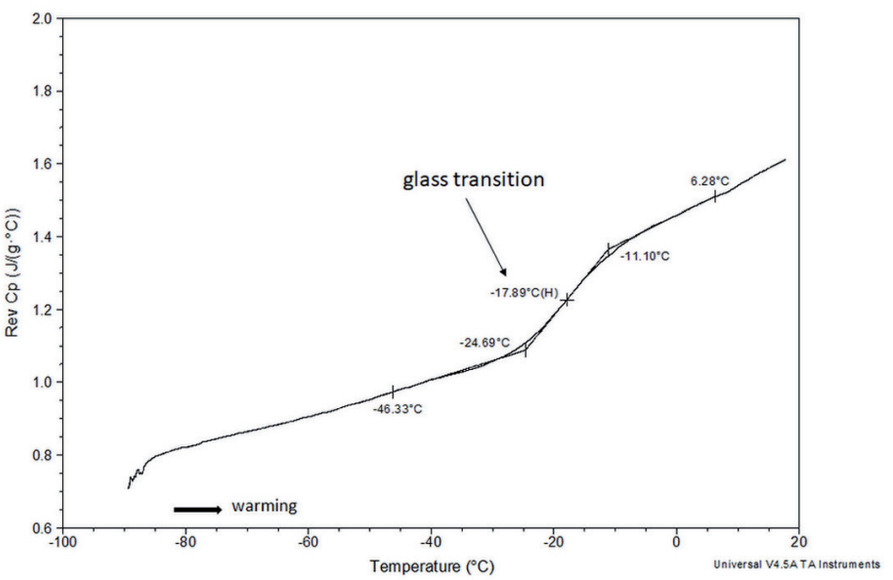

Figure 12.
Changes in reversible Cp during warming with detection of the glass transition in hop pollen (Q2000 + RCS).

TMDSC solved the limits of standard DSC method, but it has some limits too. It is time-consuming due to slower cooling/warming ramp rates. This can be partially solved by temperature modulation in narrower temperature range, which could be previously identified by a standard DSC measurement. TMDSC is sensitive to specific parameter measurement settings, which may result in failure to determine the events due to inadequate parameters of measure. Additionally, the interpretation of the results obtained can be difficult as it carries out a more detailed analysis which provides detailed results that are difficult to interpret (e.g., kinetic events).

5. Quasi-isothermal temperature-modulated DSC

QITMDSC is a specific variant of TMDSC. This method of thermal analysis is used for the direct measurement of complex Cp at given temperatures in

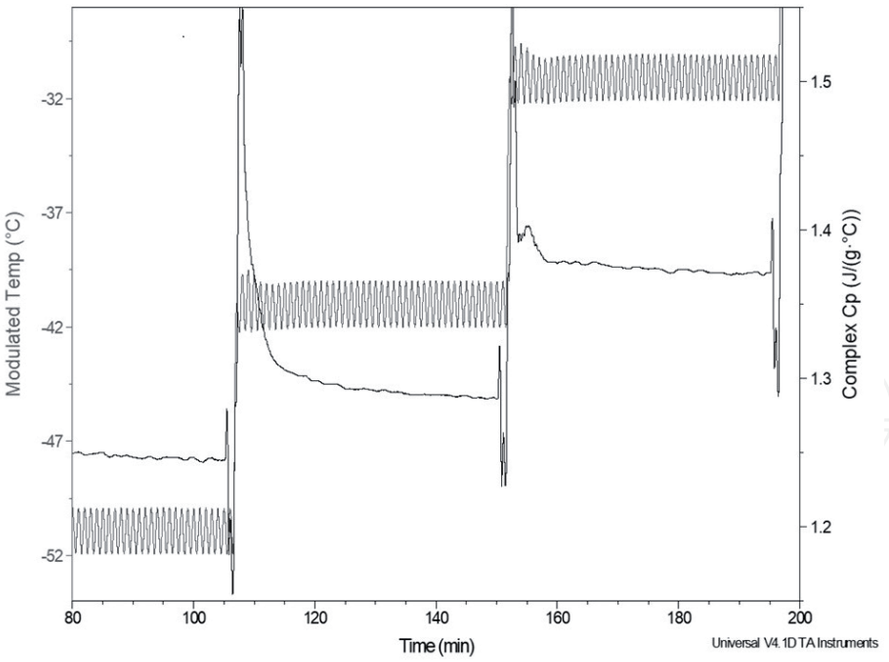

Figure 13.
Temperature modulation at a constant temperature until heat capacity equilibrates. After equilibration of heat capacity, the temperature jumps to higher temperature and modulation starts again (TA 2920 + RCS).

equilibrium conditions, at zero ramp cooling/warming rates (**Figure 13**). Hence, it is used to determine the Cp, more specifically, the thermodynamic events such as melting and glass transition.

5.1 The exact measurement of Cp under equilibrium conditions

The QITMDSC measures sample heat capacity (Cp) similarly like in the case of MDSC. That is why similar achievements can be obtained. This method can be used together with a method of matter relaxation during annealing at increasing temperature. The advantage of this method is the following measurement of the sample at stable conditions because all processes can be finished during modulation at equilibrium conditions (temperature). The glassy state is also characterized by an S-shaped curve of Cp baseline; on the other hand, melting is characterized by a peak (**Figure 14**).

5.2 Benefits and limits of TMDSC

This method is applied in the development of a cryopreservation protocol as it is useful in the determination of the state of matter and exact measurement of glass transition and melting. Additionally, it is adventitious in obtaining a more exact measurement of Cp as the Cp equilibrium conditions avoid the effects of kinetic events.

However, there are a few limitations in using QITMDSC as it is also very time-consuming, though this can be partially solved by analysis in limited temperature range, which was previously analyzed by a standard DSC or TMDSC analysis. The method is also sensitive to the parameters of measurement settings which may result in failure to determine the events due to inadequate parameters of measure. Nevertheless, as is the case with TMDSC if applied correctly, this method represents a useful tool when developing a cryopreservation protocol for vegetatively propagated plant species.

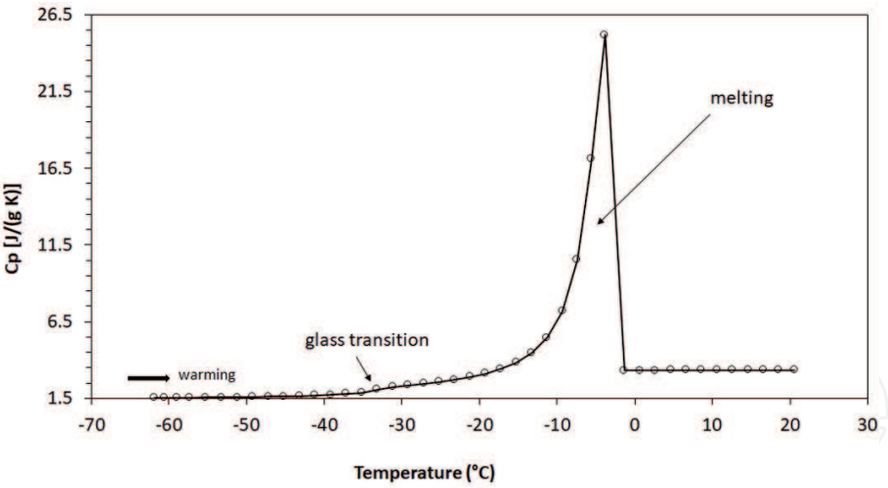

Figure 14.
Changes in Cp revealed the glass transition and the melting peak in 20% sucrose solution analyzed by QITMDSC after relaxation at −6°C (Q2000 + RCS).

6. Conclusion

Understanding the dynamics of water content in plant tissues during cooling and heating is crucial in developing a reliable cryopreservation protocol. Differential scanning calorimetry thermal analysis methods such as standard DSC, temperature-modulated DSC, and quasi-isothermal temperature-modulated DSC play a key role when cryopreserving plant material as they not only broaden the knowledge of thermal events but can also help to overcome the freezing injury during cryopreservation. The standard DSC method is recommended for routine work with known thermal properties of the sample and nonoverlapping thermal events. The MDSC method is recommended to use for plant samples with overlapping thermal events or for exact measurement of thermodynamic events. The hidden thermal events can be arisen and be separated by temperature modulation. The MDSC is also recommended to distinguish between reversing and non-reversing thermal events. The measurement of heat flow and heat capacity can be done in a single experiment. The QITMDSC is recommended to use for exact measurement of heat capacity in equilibrated conditions which can help a state of matter identification.

Acknowledgements

The authors would like to thank Mrs. Zdena Hybnerova and MS. Adrijana Cajkova for their technical help. This work is partially supported by projects QK1910277, QK1910476, and RO0418 from the Czech Ministry of Agriculture.

DOI: http://dx.doi.org/10.5772/intechopen.89723

Author details

Stacy D. Hammond Hammond, Miloš Faltus* and Jiří Zámečník
Crop Research Institute, Prague, Czech Republic

*Address all correspondence to: faltus@vurv.cz

References

[1] Benson EB, Reed BM, Brennan RM, Clacher KA, Ross DA. Use of thermal analysis in the evaluation of cryopreservation protocols for *Ribes nigrum* L germplasm. Cryo-Letters. 1996;**17**(6):347-362. ISSN: 0143-2044

[2] Grospietsch M, Stodůlková E, Zámečník J. Effect of osmotic stress on the dehydration tolerance and cryopreservation of *Solanum tuberosum* shoot tips. Cryo-Letters. 1999;**20**(6): 339-346. ISSN: 0143-2044

[3] Hirai D, Sakai A. Cryopreservation of in vitro-grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-vitrification. Potato Research. 1999;**42**(2):153-160. DOI: 10.1007/BF02358405

[4] Luyet B. 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. 1937;**204**: 1506-1508. ISSN: 0001-4036

[5] Fahy GM. Cold shock injury is a significant factor in freezing-injury—A position against. Cryobiology. 1985;**22**(6):628-628. DOI: 10.1016/0011-2240(85)90111-7

[6] Langis R, Steponkus PL. Cryopreservation of rye protoplasts by vitrification. Plant Physiology. 1990;**92**(3):666-671. DOI: 10.1104/pp.92.3.666

[7] Uragami A, Sakai A, Nagai M, Takahashi T. Survival of cultured-cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification. Plant Cell Reports. 1989;**8**(7):418-421. DOI: 10.1007/BF00270083

[8] Towill LE. Cryopreservation of isolated mint shoot tips by vitrification. Plant Cell Reports. 1990;**9**(4):178-180. DOI: 10.1007/BF00232174

[9] Suzuki M, Tandon P, Ishikawa M, Toyomasu T. Development of a new vitrification solution, VSL, and its application to the cryopreservation of gentian axillary buds. Plant Biotechnology Reports. 2008;**2**(2):123-131. DOI: 10.1007/s11816-008-0056-5

[10] Nishizawa S, Sakai A, Amano Y, Matsuzawa T. Cryopreservation of Asparagus (*Asparagus officinalis* L) embryogenic suspension cells and subsequent plant regeneration by vitrification. Plant Science. 1993;**91**(1):67-73. DOI: 10.1016/0168-9452(93)90189-7

[11] Liu HQ, Yu WG, Dai JX, Gong QH, Yang KF, Lu XZ. Cryopreservation of protoplasts of the alga *Porphyra yezoensis* by vitrification. Plant Science. 2004;**166**(1):97-102. DOI: 10.1016/j.plantsci.2003.08.014

[12] Liu YG, Wang XY, Liu LX. Analysis of genetic variation in surviving apple shoots following cryopreservation by vitrification. Plant Science. 2004;**166**(3):677-685. DOI: 10.1016/j.plantsci.2003.11.003

[13] Martinez D, Revilla MA, Espina A, Garcia JR. Differential scanning calorimetry applied to the storage at ultra-low temperatures of olive and hop in vitro grown shoot-tips. Thermochimica Acta. 2000;**349**:147-151. DOI: 10.1016/S0040-6031(99)00508-0

[14] Köpnick C, Grübe M, Stock J, Senula A, Mock H-P, Nagel M. Changes of soluble sugars and ATP content during DMSO droplet freezing and PVS3 droplet vitrification of potato shoot tips. Cryobiology. 2018;**85**:79-88. DOI: 10.1016/j.cryobiol.2018.09.005

[15] Nadarajan J, Akter A. Optimization of cryopreservation protocols for zygotic embryos of *Citrus reticulata*. Acta Horticulturae. 2019:137-144. DOI: 10.17660/ActaHortic.2019.1234.18

[16] Zámečník J, Faltus M, Bilavčík A. Application of differential thermal analysis for the improved regeneration after cryopreservation of vegetatively-propagated plants. Acta Horticulturae. 2019;**1234**:57-64. DOI: 10.17660/ActaHortic.2019.1234.7

[17] Vertucci CW, Stushnoff C. The state of water in acclimating vegetative buds of apple and Amelanchier and its relationship to winter hardiness. Physiologia Plantarum. 1992;**86**:503-511. ISSN: 0031-9317

[18] Wang MR, Chen L, Teixeira da Silva JA, Volk GM, Wang Q-C. Cryobiotechnology of apple (Malus spp.): Development, progress and future prospects. Plant Cell Reports. 2018;**37**:689. DOI: 10.1007/s00299-018-2249-x

[19] Keller ERJ, Senula A. Recent aspects of Allium cryopreservation in the federal German genebank. Acta Horticulturae. 2016;**1143**:35-44. DOI: 10.17660/ActaHortic.2016.1143.6

[20] Buitink J, Walters-Vertucci C, Hoekstra FA, Leprince O. Calorimetric properties of dehydrating pollen: Analysis of a desiccation tolerant and an intolerant species. Plant Physiology. 1996;**111**:235-242. DOI: 10.1104/pp.111.1.235

[21] Keller ERJ, Senula A. Recent aspects of Allium cryopreservation in the federal German genebank. In: 7th International Symposium on Edible Alliaceae; 21-25 May 2015; Nigde, Turkey. International Society for Horticultural Science. Acta Horticulturae. 2016;**1143**:35-44. DOI: 10.17660/ActaHortic.2016.1143.6

[22] Vertucci CW. Effects of cooling rate on seeds exposed to liquid nitrogen temperatures. Plant Physiology. 1989;**90**:1478-1485. DOI: 10.1104/pp.90.4.1478

[23] Vertucci CW. Relationship between thermal transitions and freezing injury in pea and soybean seeds. Plant Physiology. 1989;**90**:1121-1128. DOI: 10.1104/pp.90.3.1121

[24] Prasongsom S, Thammasiri K, Narangajavana J, Thitamadee S, Chuenboonngarm N, Panvisavas N. Vitrification-based cryopreservation of *Dendrobium cruentum* Rchb. f. seeds. Acta Horticulturae. 2019;**1234**:157-166. DOI: 10.17660/ActaHortic.2019.1234.21

[25] Kaczmarczyk A, Rokka VM, Keller ERJ. Potato shoot tip cryopreservation. A review. Potato Research. 2011;**54**:45-79. DOI: 10.1007/s11540-010-9169-7

[26] Zamecnikova J, Fernandez E, Viehmannova I, Zamecnik J, Faltus M. Preparation of shoot tips by sucrose and dehydration pre-treatment of *Ullucus tuberosus* CAL. For cryopreservation. Acta Horticulturae. 2011;**908**:331-338. DOI: 10.17660/ActaHortic.2011.908.44

[27] Gonzalez-Benito ME, Mendoza-Condori VH, Molina-Garcia AD. Cryopreservation of in vitro shoot apices of *Oxalis tuberosa* Mol. Cryo Letters. 2007;**28**:23-32. ISSN: 0143-2044

[28] Zamecnik J, Faltus M, Kotkova R, Hejnak V. Glass transition determination in allium shoot tips after dehydration. Acta Horticulturae. 2011;**908**:33-38. DOI: 10.17660/ActaHortic.2011.908.1

[29] Lynch PT, Graham RS, Zámečník J, Harding K. Optimization of water content for the cryopreservation of *Allium sativum* in vitro cultures by encapsulation-dehydration. Cryo

Letters. 2016;**37**:308-317. ISSN: 0143-2044

[30] Vogiatzi C, Grout BW, Wetten A, Toldam-Andersen BT. Cryopreservation of winter-dormant apple buds: II - tissue water status after desiccation at −4C and before further cooling. Cryo Letters. 2011;**32**:367-376. ISSN: 0143-2044

[31] Bachiri Y, Song GQ, Plessis P, Shoar-Ghaffari A, Rekab T, Morisset C. Routine cryopreservation of kiwifruit (Actinidia spp) germplasm by encapsulation-dehydration: Importance of plant growth regulators. Cryo Letters. 2001;**22**:61-74. ISSN: 0143-2044