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Glass transition and heat capacitybehaviors of plant vitrification solutions

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Graphical abstract

T_G and ΔCp measurement in plant cryopreservation solutions.

Highlights

- T_G and associated ΔCp measurement in Plant Vitrification Solutions (PVS)
- The T_G of five PVS can be related to their different composition, especially to its water content
- T_G of PVS and its associated ΔCp are not altered (or only very slightly) by a wide range of cooling and warming rates
- Consequently, T_G of these PVS do not significantly change with cryopreservation methods with different cooling/warming rates

ABSTRACT

Differential scanning calorimetry (DSC) was employed to investigate the vitrification and annealing behaviors of the most commonly used plant vitrification solutions (PVS). These solutions are employed to protect plant tissues towards ice formation and freeze injury, and help to the vitrification of these tissues, by globally reducing the intracellular fluids mobility. Glass transition temperatures ($\mathbf{T}_g$) and heat capacity increments ($\Delta\mathbf{Cp}$) were determined for five solutions PVS1, PVS2, PVS2 mod, PVS3 and PVS3 mod, with different composition, and a range of cooling and warming rates was employed. Glass transitions showed clear and consistent temperature differences within vitrification solutions, which could be related to composition and water content. Roughly, two sets of $\mathbf{T}_G$ values were obtained, those for PVS1 and 2, at -112 ºC and -114 ºC, respectively, and those for PSV3, at -90 ºC. The observed $\mathbf{T}_g$ and $\Delta\mathbf{Cp}$, unexpectedly, did not significantly change within a wide range of cooling rates (from 5 ºC min^{-1} to liquid nitrogen quenching) and warming rates (from 5 to 20 ºC). Garlic shoot tips cryopreserved after the droplet method produced a similar result to that of the vitrification solutions employed. After quench cooling to temperatures below $\mathbf{T}_g$, repeated excursions to higher temperatures were made and the cooling and warming $\mathbf{T}_g$ were recorded. These treatments had little or no effect over the PVS solutions $\mathbf{T}_g$, which remained practically constant. A direct practical consequence is that the plant vitrification solutions glass transition temperature does not significantly change with cryopreservation methods based on either direct plunging of samples into liquid nitrogen or employing closed cryovials.

1. Introduction

Glass, the state of matter where molecular mobility is so reduced that most physicochemical processes (including ice formation) are actually detained, is a basic state of matter for cryopreservation. The temperature at which glass initially forms from supercooled liquid is

known as the glass transition temperature ($\mathbf{T_G}$). It is rather a range of temperatures than a single defined one. The glass transition is also characterized by a change in heat capacity ($\Delta\mathbf{C_p}$), which is measured as the baseline difference at the transition inflection point. At temperatures above $\mathbf{T_G}$, there is an increase in molecular mobility and a decrease in viscosity which enables the crystallization of water, if sufficient time to carry out the process is granted [1]. Per contra, when temperature is below the glass transition, the system can be considered stable. Most physical and chemical processes (especially those that are diffusion driven) are impeded, in a physically accessible time frame. Actually, there is movement in the glassy state: many reports describe the existence of a significant degree of short range mobility, associated to solvent molecules (water, in this case) (for example Ablett et al. [2] and Le Meste et al. [3]). Within the very field of plant cryopreservation, a reduced but not null molecular movement degree has been found in intracellular glasses, investigated by different physical techniques [4-7]. However, the relatively large-scale molecular reorganizations required for ice crystal nucleation are certainly not possible in these conditions of greatly reduced molecular mobility [8].

Consequently, glassy state allows living tissues preservation with no or very limited physicochemical changes and completely free of ice formation, which is associated with lethal freeze injury [9,10]. Since the knowledge that cells treated with specific cryoprotecting solutions survive exposure to cryogenic temperatures was achieved, numerous variations on solution composition were developed for plant cells [11]. Different vitrification solutions were developed by various research teams worldwide [12,13]. Sakai's group developed the PVS (Plant Vitrification Solution) series. PVS1 has been employed for work with asparagus cell suspensions [14,15]. PVS2, originally developed for treating citrus cell suspensions [12], has been successfully used for different explants of around 200 species [16], including garlic [11,17]. PVS3 has been notably employed with wasabi [18], asparagus [19] and garlic [20]

shoot tips. The cryopreservation of garlic germplasm, an economically important crop, has been profusely studied by several authors using different approaches [11,17,20-22].

The glass transition temperature of aqueous systems is very sensitive to the proportion of water and other small molecules, and small changes in compositions can give rise to large variations in $\mathbf{T}_G$. When taking advantage of the glassy state for preservation purposes, often the difficulty lies in the prediction and control of this temperature. Another problem is how to reach temperatures below $\mathbf{T}_G$, from room temperature, without ice being formed during the cooling process (the same problem arises when warming specimens to room temperature) [1,23]. The probability of ice formation increases with the time that the system is at a temperature comprised between $\mathbf{T}_f$ (the equilibrium freezing temperature) and $\mathbf{T}_G$. Actually, the ice nucleation temperature should be considered, instead of $\mathbf{T}_f$. This nucleation temperature has not a fixed value, being dependent on Brownian movement, but is often placed well below $\mathbf{T}_f$. On the other hand, the region "close" above $\mathbf{T}_G$ is considered too viscous for anything like ice nucleation to happen, in a short time frame. A zone of 20ºC above $\mathbf{T}_G$ is often accepted as having a very low probability of ice formation [24].

The glass transition is not a phase transformation in the full thermodynamic sense. Its occurrence is determined by the history of the material, and is dependent on the exact conditions of the experiment [25]. The change between liquid and vitreous state has also been described as a second order phase change, not characterized by an enthalpy increment as first class changes are, but by a step in some of the physical properties, such as the heat capacity, specific volume and apparent viscosity [26]. Differential scanning calorimetry (DSC) is a powerful tool to investigate glass transition and heat capacity behaviors of plant vitrification solutions [27]. A model plant system (garlic) was included in the experimental design, to compare the behavior of the pure solutions and that of a real system. Garlic shoot tips

cryopreservation had been studied previously, using PVS2 [11] and PVS3 [28] cryopreservation solutions.

The present study aimed the characterization of the calorimetric properties of the most common plant vitrification solutions under the same range of cooling and warming rates, at conditions relevant to their use as cryopreservation agents. The characterization of the calorimetric properties of garlic shoot tips treated with one of such solutions (PVS3) is also included.

2. Materials and methods

2.1. Standard compounds

Dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG) of chromatographic grade were purchased from Sigma Aldrich (Heidelberg, Germany); glycerol, sorbitol and sucrose used were manufactured by Penta (Chrudim, Czech Republic) and isothiazolinone by Schulke & Mayr (Norderstedt, Germany). All other chemicals used were of the highest commercially available purity.

2.2. Solutions

The vitrification solutions were (**Table 1**): (1) PVS1 (Plant Vitrification Solution 1: 19% w/v glycerol, 13% w/v EG, 13% w/v PG, 6% w/v DMSO in half-strength MS liquid medium + 0.5 M sorbitol) [14], (2) PVS2 (30% w/v glycerol, 15% w/v EG, 15% w/v DMSO, and 0.4 M sucrose in half-strength MS liquid medium) [12], (3) modified PVS2 (PVS2 mod: 37.8% w/v glycerol, 16.7% w/v EG, 16.5% w/v DMSO and 0.4 M sucrose in half-strength MS liquid), (4) PVS3 (50% w/v glycerol, 50% w/v sucrose in water) [19], (5) modified PVS3 (PVS3 mod: 50% w/v glycerol, 50% w/v sucrose, 5% DMSO in water) [19].

2.3. Plant material

Experiments were carried out with *Alium sativum* L. *sativum* cv. ´Djambul 2´. Microbulbils (small areal bulbs formed together with flower buds) were rinsed with 1% isothiazolinone solution for two hours. Subsequently, the shoot apex (1 mm) in each microbulbil was excised with a scalpel blade and forceps. Shoot apices were placed in 75% isothiazolinone solution for 2 s and then transferred to 1% isothiazolinone solution for 10 min. Finally, batches of 20 apices were directly placed onto MS medium [29] with 10% sucrose overnight.

Excised shoot tips were immersed into the loading solution (13.7% w/v sucrose + 18.4% w/v glycerol) [12] for 20 min at 25ºC. Shoot tips were immersed in PVS3 at 25ºC for 2 h [30].

2.4. Differential scanning calorimetry conditions

Thermal processes in vitrification solutions were measured using a TA 2920 DSC (TA Instruments, New Castle, DE, USA). Hermetic aluminum pans (TA) were used in all DSC experiments and an empty pan was used as reference. The furnace block of DSC was flushed with dry nitrogen gas to avoid condensation of moisture from the air. Helium gas (99.999%) was used as sample purge at a rate of about 33 ml min^{-1}. The temperature scale of the instrument was calibrated with cyclohexane (-87°C), mercury (-38.87°C), water (0.01°C) and indium (156.6°C) and with heat fusion of water (334 J g^{-1}). All calibrations were performed by using scanning rates of 5, 10 and 20°C min^{-1}. Calorimetric data were collected from two replicates per treatment.

Samples within pans were either approx. 10 mg of vitrification solutions or three garlic shoot tips, immediately after treatment with the vitrification solution PVS3. In work with shoot tips, pans were weighed before and after DSC; in the second case pans were punctured and oven-dried (~85°C, 72 h), to obtain the water content. Calorimetric data were collected from two replicates per treatment.

2.5. Glass transition characterization for PVS

The glass transition parameters ($\mathbf{T_G}$ and $\mathbf{\Delta Cp}$) were determined for the different vitrification solutions considered, in both cooling and warming scans. Pans with samples were cooled at standard rates (10ºC min^{-1}) from 30ºC to -145ºC. After 5 min equilibration at this temperature, they were warmed back to 30ºC, also at 10ºC min^{-1}. This cycle was repeated four times. Two different samples were studied for each solution.

2.6. Cooling rate test

For this test, cooling was performed at different rates: either using the calorimeter control (5, 10 and 20°C min^{-1}), or, for higher rates, by quickly immersing the closed pan with the sample in liquid nitrogen (LN), either naked or previously included inside a cryovial (approximately 6600°C min^{-1} and 160°C min^{-1}, respectively [31]). The pan was then transferred to the calorimeter sample chamber, pre-cooled to -150°C, where a short equilibration time was allowed. Glass transition temperature and the corresponding heat capacity change were observed upon warming from -145°C to room temperature, at a standard warming rate of 10°C min^{-1}. The samples were either vitrification solutions or garlic shoot tips, after treatment with the vitrification solution PVS3.

2.7. Warming rate test

For investigating the effect of the warming rate, cooling was performed at two fixed rates: 10°C min^{-1} (using the calorimeter control) or by quenching in LN (without cryovial). Quenched pans were transferred to the calorimeter sample chamber, pre-cooled to -150°C, where a short equilibration time was allowed. Glass transition temperature and the corresponding heat capacity change were measured upon warming pans with samples from -

145°C to room temperature, at different warming rates: 5, 10 and 20°C min^{-1}. The samples were either vitrification solutions or garlic shoot tips, after treatment with the vitrification solution PVS3.

Crystallinity, for garlic shoot tips was evaluated using TA proprietary software (TA Instruments, undated), using a value for the specific fusion heat of water of 334 J g^{-1}. The routine basically evaluates the thermal event enthalpy and, by comparing with the water specific fusion heat and considering the water content of the sample (obtained by differential weighing, after drying in an oven after the DSC experiment), calculates the fraction of water that had crystallized into ice.

2.8. Statistical analysis

The results were analyzed by analysis of variance (ANOVA), and means were compared by Duncan's multiple range test ($P \leq 0.05$) using STATISTICA version 10, (StatSoft, Inc., 2011).

3. Results and Discussion

3.1. Vitrification behavior of different vitrification solutions

Glass transition parameters were determined for the five PVS employed, at standard rates of 10ºC min^{-1} (**Table 2**). Glass transitions temperatures were fairly reproducible and showed clear and consistent differences among vitrification solutions (confirmed by the statistical analysis), which were related to different composition and water contents (**Table 1**). $\mathbf{T}_G$ values calculated for each solution were identified by the statistical analysis as distinct (**Table 2**, $P< 0.001$). The value of $\mathbf{T}_G$ generally decreased as the water content of solutions with similar chemical composition increased. For example, $\mathbf{T}_G$ tended to decrease as the water content of a particular plant vitrification solution increased (i.e., PVS2 vs. PVS2mod, PVS3

vs. PVS3mod). But also $\mathbf{T}_G$ tended to decrease as the water content of plant vitrification solutions based in glycerol and sucrose increased. A minimum square regression to a straight line yielded a high regression coefficient (R2 =0.991795) for $\mathbf{T}_G$ values of PVS2, PVS2mod, PVS3 and PVS3mod. PVS1 was excluded from this regression since glycerol and sucrose are not present in its chemical composition and, differently than PVS2 or 3, includes sorbitol and PG in its composition.

The general role of water as plasticizer agents and their effect reducing the value of $\mathbf{T}_G$ have been extensively studied (i.e. [32,33]. These vitrification solutions contained large amounts of solutes able to form hydrogen bonds with water. The number of water molecules available per each solute molecule was relatively low and even lower the number of water molecules per potential hydrogen bond in solute molecules. Nevertheless, in such concentrated and complex mixtures not all these potential bonds could be expected to bind water, as many intersolute (and intramolecular) bonds would arise for molecular formulas of the components of the cryopreservation solutions and their molar composition.

The different $\mathbf{T}_G$ found could be divided into two groups: one centered around -112ºC (PVS1, PVS2 and PVS2 mod) and the other at -89ºC (PVS3 and PVS3 mod). The heat capacity changes measured were less distinct than the corresponding $\mathbf{T}_G$ values, but a decrease with the water content in solutions could be appreciated and these data can be fitted to a straight line (R^2= 0.944513, which improves to 0.992353 upon discarding the value for PVS1). However, PVS solutions could be distributed according to $\Delta\mathbf{Cp}$ in the same groups. The prediction of glass transition behavior for complex mixtures as those studied here, is, currently, not a solved problem (see, for example, Angell et al. [34]). Nevertheless, water content seems to be the dominant factor over the solute composition.

Very few data on glass transition parameters of PVS have been reported. The initial PVS2 publication [12] reported a $\mathbf{T}_G$ value of -115ºC (equal for both cooling and warming scans),

within experimental error of our data. It must be noted that, in the same publication, the existence (upon warming at 10ºC min^{-1}, following 80ºC min^{-1} cooling) of a devitrification event (at -75ºC), subsequently followed by melting (at -36ºC) was reported. After extensive, repeated and reproducible DSC experiments, we have been unable to find any ice formation event, at any of the combinations of cooling and warming rates considered, and for any of the PVS studied. The reported devitrification event took place upon warming at 10ºC min^{-1}, twice as fast as our 5ºC min^{-1} slowest experiment (where ice formation would have been more likely). PVS3 was also previously reported to have a $\mathbf{T}_G$ in good agreement with those reported here [28].

Pure water glass transition temperature, though difficult to physically measure, is believed to take place at 135K (-138ºC) [35,36], actually not so far away from the $\mathbf{T}_G$ found here for PVS solutions. Other authors even rise this temperature to 165 K (-108ºC) [37,38], or even higher [39,40]. Clearly, the question is far from being settled.

Binary mixtures (water and a single solute) can partly explain the $\mathbf{T}_G$ values reported here for PVS. Sucrose aqueous solutions with a 40-60% (w/w) solute content, present glass transition temperatures ranging from -110ºC to -86ºC [41], comparable with those obtained in this work (PVS3 and PVS3-mod have approx. 40% w/w sucrose content). Meanwhile, glycerol, which is present in the aforesaid solutions also at ≈ 40% w/w, but at ≈ 30% w/w in PVS2 and PVS2-mod, would, in binary aqueous systems, show a $\mathbf{T}_G$ at approx. -100ºC and -110ºC, respectively [42]. PVS1, lacking glycerol and sucrose, has a combined ethylen and propylen glycol content of ≈ 25% w/w. A 25% w/w ethylen glycol water solution would give raise to a $\mathbf{T}_G$ between -130ºC and -135ºC [42]. A correct prediction of the glass transition temperatures for PVS from composition data would be a more complex matter, though.

3.2. Effect of cooling rate on vitrification behavior

Vitrification in cryopreservation protocols is achieved, without sophisticated cooling equipment, by simply plunging specimens into LN after a set of physicochemical treatments, designed to increase their cytoplasmatic microviscosity and enhance tissue resistance to cold and dehydration. Fast cooling is required to achieve vitrification avoiding ice crystal formation [43]. In a similar way, warming after the storage period must be carried out quickly, and it is practically performed by plunging samples (naked or inside cryovials) directly from LN into a water bath or warm culture medium [31].

Thermograms showing details the effect of cooling rate on the vitrification behavior of PVS3 are shown in **Figure 1**. The glass transition recorded in the warming scan (always carried out at 10°C min^{-1}) was similar both in temperature and heat capacity change. The width of the glass transition process was estimated to be on an average of 7ºC.

$\mathbf{T_G}$ and $\mathbf{\Delta Cp}$ were measured for the five solutions studied and are shown in **Figure 2**. ANOVA indicated that the vitrification solution had a significant effect ($p < 0.001$) on $\mathbf{T_G}$, while cooling rate and the interaction of both variables/factors did not. The $\mathbf{T_G}$ of a solution did not significantly change within a wide range of cooling rates (from 5 to 20°C min^{-1}, under the DSC control, and the faster resulting from plunging the sample pan into LN, with the aluminum pan naked or inside a cryovial). The cooling rate did not influence the glass transition region, as compared with the width of the whole transition interval (**Figure 1**). The mean $\mathbf{T_G}$ for each PVS (calculated with the data from all cooling rates) were significantly different among them (according to Duncan`s test, $p< 0.05$): PVS1 -112.4210 ± 0.3656, PVS2 -114.5770± 1.01569, PVS2-mod -109.4910 ± 0.56078, PVS3 -90.0550± 0.82682 ; PVS3-mod -87.3010±1.25750.

Although $\mathbf{\Delta C_p}$ values show a larger variation, no clear trend with the cooling rate could be observed (**Figure 2**). ANOVA indicated that cooling rate did not have a significant effect on $\mathbf{\Delta C_p}$, while the vitrification solution and the interaction of solution rate did ($p < 0.001$).

Therefore, statistical analysis on both $\mathbf{T_G}$ and $\mathbf{\Delta C_p}$, showed that the differences observed among cooling rates were not significant. This was an unexpected finding, as both $\mathbf{T_G}$ and the $\mathbf{\Delta C_p}$ are generally considered to be dependent on cooling rate (e.g. [4,44]). After this, the slower a liquid is cooled, the longer time would be available for configuration of samples at each temperature, and hence the colder it could become before falling out of liquid-state equilibrium. This would be reflected in $\mathbf{T_G}$ increasing with cooling rate [45,46], as the properties of the glass depend on the process by which it is formed. In practice, the reported dependence of $\mathbf{T_G}$ on the cooling rate is weak: $\mathbf{T_G}$ changes by 3–5°C when the cooling rate changes by an order of magnitude [44,47].

Possible explanations for the discrepancy between the small variation (1.2ºC) reported here and the expected value (3-5ºC per rate magnitude order corresponds to 9-15ºC, for the more than three orders between 5 and 6600ºC min^{-1} of our experiments) may lie on that the experimental observations in which $\mathbf{T_G}$ undergoes these changes with cooling rate refer in most cases to much simpler systems, either pure liquids or binary mixtures. These ternary, quaternary and pentary solutions may present a compensating behavior, with different components having contributions of different sign to the glass transition displacement with cooling rate.

Another explanation may lay on the fact that, in the present work, the reported glass transition parameters measured at fast cooling rates were evaluated always at the ensuing warming scan. Actually, very few experimental techniques can perform a proper glass transition measurement while cooling at fast rate. Faster cooling would cause the glass transition to take place at earlier, higher temperatures (giving rise to a less stable glass). Upon warming, the glass transition would occur as soon as the lower $\mathbf{T_G}$ (of the more stable glass, found at slow warming processes) was reached. In that way, the influence of the previous cooling rate would be ignored.

Cooling rates present a wide variation in practical plant cryopreservation, though they have not been very frequently accurately measured. They range from the very slow processes of those methods relying on extracellular freezing for induced intracellular vitrification (using temperature control equipment, with rates as low as 0.3ºC min^{-1}) [48], to those of he vitrification method (approx. 2.3°C s^{-1}), the encapsulation-dehydration protocol (approx. 2.7°C s^{-1}) and the faster droplet-vitrification method (110ºC s^{-1}) [31,48-50]. Other faster approaches have been tested, such as those avoiding the Leidenfrost effect that limits the cooling rate in LN by using other cryogenic fluids, such as liquid propane or ethane, or even using mechanical implements to make faster the immersion of the sample in the cryogenic medium [11,50]. The two quench cooling procedures used here had rates similar to those of the droplet-vitrification (naked DSC pan) and the encapsulation-dehydration method (inside the cryovial), i.e. approximately 110ºC s^{-1} (6600ºC min^{-1}), and 2.7ºC s^{-1} (160ºC min^{-1}), respectively.

The behavior of the vitrification parameters of garlic shoot tips in the last stage of the cryopreservation protocol (after treatment with PVS3) was different from that of the vitrification solutions (**Figure 2)**. Shoot tips present a much more marked dependence of both $\mathbf{T}_G$ and $\Delta\mathbf{C}_p$ with the cooling rate than solutions. This may be due to the fact that the shoot tips have higher cytoplasmic water content or to the presence of other cellular components, apart form their structural and compartmentalization elements.

The glass transition parameters obtained here differ from those of other works, which had been carried out in different conditions: the $\mathbf{T}_G$ reported for garlic studies using PVS2 is approximately -115ºC [11]. This is a very close data to the PVS2 solution $\mathbf{T}_G$ data reported in this work (-114.3ºC). Accordingly, our values (ranging from -90 to -100ºC for different rates), are close to the -89.85ºC found for PVS3. The $\Delta\mathbf{C}_p$ data for garlic, showing a strong variation in the present study, are not too different from those reported using PVS2 [11].

3.3. Effect of warming rate on vitrification behavior

The glass transition parameters were measured for different warming rates after using two different cooling rates: 10°C min^{-1} (**Figure 3a** and **c**) and LN quenching (**Figure 3b** and **d**). The variations in both $\mathbf{T}_G$ and $\Delta\mathbf{C}_p$ were not significant and, again, showed no dependence of the warming rat. A clear relation between $\mathbf{T}_G$ and the solution composition, for all the cooling and warming conditions studied, could be observed, with a similar response of the group PVS1-PVS2-PVS2 mod, on one hand and the group PVS3-PVS3 mod, on the other (data not shown). The dependence of $\Delta\mathbf{C}_p$ with composition for the different warming and cooling rates tested was more obscure.

Practical warming rate values for cryopreservation are of the same order of those reported for cooling rates, as most cases heat exchange is driven by stirring the sample (naked, encapsulated or inside a cryovial) in a warm water bath or culture medium, being the temperature gradients induced comparable to those created by plunging room temperature specimens into LN. Nonetheless, warming rates slightly lower than those found for cooling have been reported [31,49]: approx. 1.8, 2 and 40°C s^{-1}, for mint shoot tips following the vitrification, encapsulation-dehydration and droplet-vitrification protocols, respectively.

3.4. Crystallinity of garlic shoot tips

Garlic shoot tips subjected to the vitrification procedure exhibited both freezing and glass transition events when cooled slowly (10ºC min^{-1}) (**Figure 4**). Other systems treated by the vitrification method did not produce water freezing when, in the last step of the protocol, were cooled at this slow rate. This is the case of mint shoot tips [51] and this might be due to their smaller size, when compared with garlic shoot tips. Mint shoot tip average weight after the vitrification dehydration step was 0.7 mg [51], while garlic shoot tips in the same stage

weighed 3.9 ± 0.4 mg. Sample size is one of the most important determinants of vitrification probability [25]. Other differences, such as water content or permeability to solutes, may sustain alternative explanations.

The presence of this (always very small) melting endotherm allows calculation of the fraction of crystallized water. The onset of the melting process and the crystallinity calculated after the TA proprietary software, are presented in **Table 3** for different warming rates. The crystallinity decreased with increasing warming rates, while the transition onset grew. The corresponding glass transition temperatures were almost constant with warming rate. In similar systems, it has been reported that ΔC_p decreases while crystallinity increases [52], which agrees with the observations of **Figure 4** and **Table 3.** Although, at quenching rates (those employed in practical cryopreservation) there was no formation of ice, these findings could imply a potential amount of freezable water that could result in ice formation.

Garlic shoot tips cryopreservation has previously been studied employing PVS2 instead of PVS3 [11]. After 15 min exposure to PVS2, garlic shoot tips were reported to show a melting endotherm centered at -50ºC, corresponding to approximately 12% crystallinity (at a scan rate of 10ºC min^{-1}). 30 min exposure, yielding the maximum plant recovery after cryopreservation, showed no endotherm [11]. The freezing (observed at the lower nucleation temperature) and melting events of garlic shoot tips at this work (employing PVS3) **Figure 4** and **Table 3**, were present only at the 10ºC min^{-1} rate. In conditions customarily employed for successful cryopreservation (including quench cooling rates) both freezing and melting events were not observed. The crystallinity degree found was small (2-0.4 %, depending on the warming rate). As the temperature of these events was higher (-15 to -20ºC, **Table 3**) than that reported by Volk and coworkers [11], a possible interpretation is that these events might correspond to a separate water fraction from that required for germplasm viability. Alternatively, the higher temperature of the melting event could be the result of using PVS3 rather than PVS2, and a

border condition, where ice is formed only at low cooling rates but not at higher quenching rates, could have been reached.

Conclusion

T_G and ΔCp of plant vitrification solutions did not significantly change when the cryopreservation methods based on either direct plunging of samples into liquid nitrogen or plunging of samples in closed cryovials, or even slower methods, were used. We can conclude that the T_G of commonly employed PVSs did not change with the wide range of cooling and warming rates. The behavior of cryopreserved tissues might be, though, different.

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Figure 1. PVS3 thermograms showing the glass transition, performed at a warming rate of 10ºC min^{-1} and after cooling at different rates. Quenching: direct immersion of the DSC pan in LN; cryovial: pan included in a cryovial and then in LN. White arrows mark the glass transition inflection point.

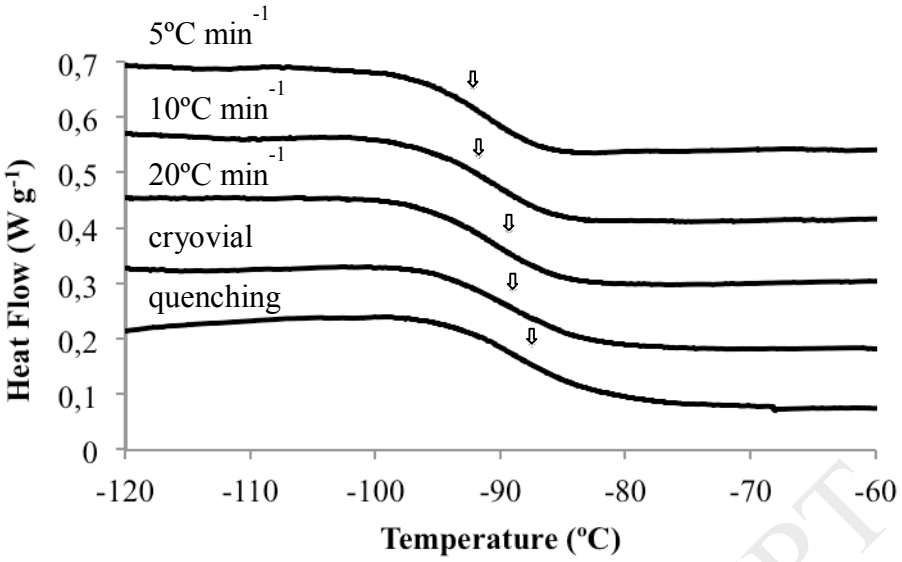

Figure 2. Glass transition temperatures (**a**) and glass transition heat capacity changes (**b**) for the different PVS and garlic shoot tips (after treatment with PVS3), obtained in warming (10ºC min^{-1}) DSC experiments, after being cooled at different rates.***** Rate not tested for garlic samples.Mean values of T_G and ΔCpfrom garlic samples with the same letter are not significant different according to the Duncan's Multiple Range Test at alpha = 0.05.Bars: standard deviation.

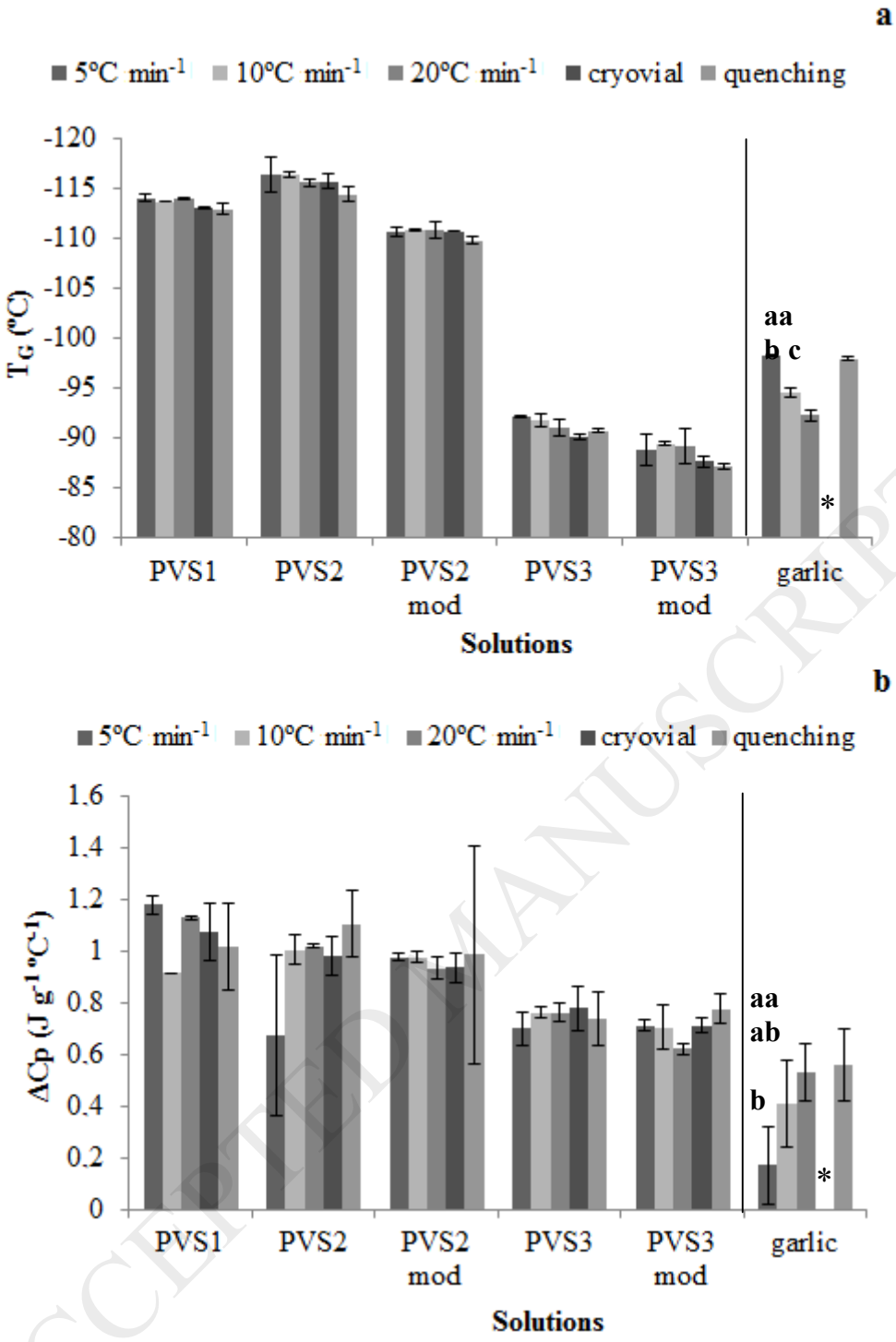

Figure 3. Glass transition temperature (**a** and**b**) and heat capacity (**c** and **d**) obtained by DSC during warming at different rates (5, 10 or 20°C min^{-1}). DSC pans had been cooled either at 10°C min^{-1} in the calorimeter (**a** and**c)** or by quenching in LN (**b** and **d**). Bars:standard deviation.

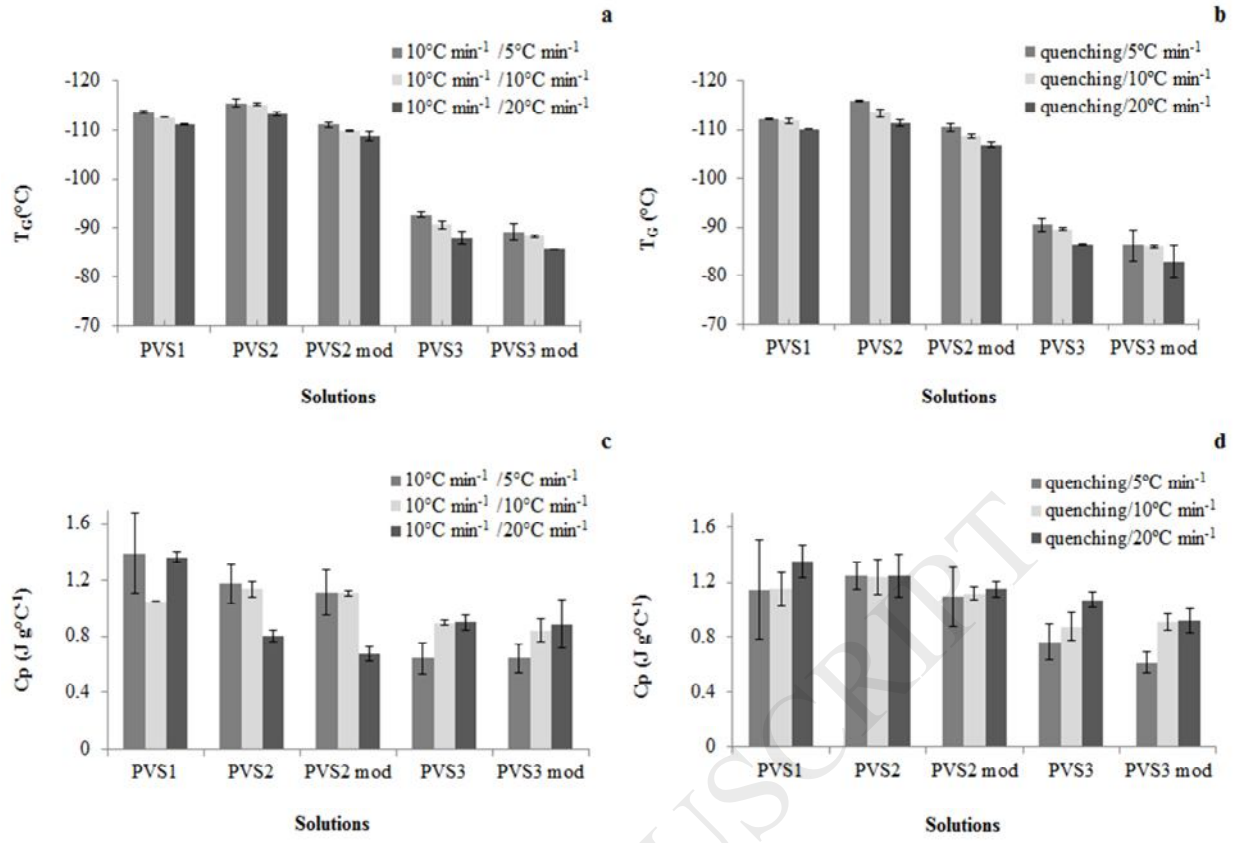

Figure 4. Typical scan of shoot tips of garlic after treatment with PVS3 for 2 h. Both cooling and warming rates were 10ºC min^{-1}. The black arrows mark to the glass transition inflection point in cooling and warming scans, while gray arrows signal the freezing and melting events.

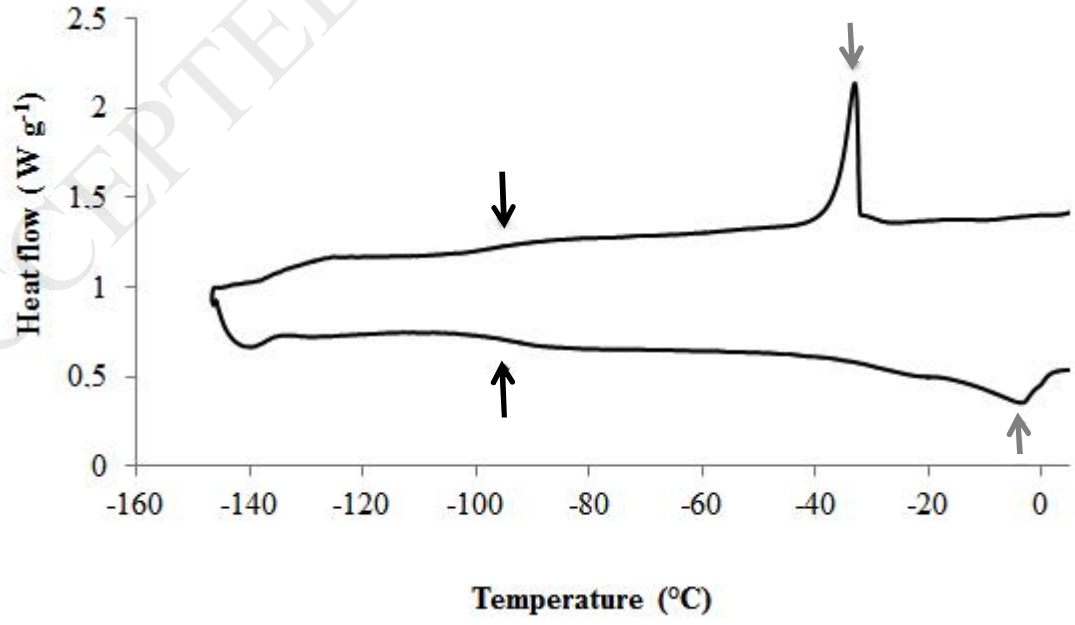

Table 1. Composition of vitrification solutions.

Solution	Component (% w v^{-1})						
	Sorbitol	EG	DMSO	PG	Glycerol	Sucrose	Water
PVS1	9.1	13	6	13	-	-	64.1
PVS2	-	15	15	-	30	13.7	40.3
PVS2 mod	-	16.5	16.5	-	37.8	13.7	31.3
PVS3	-	-	-	-	50	50	28.6
PVS3 mod	-	-	5	-	50	50	24.1

Table 2. Glass transition parameters of different plant vitrification solutions (cooling and warming rates were both 10°C min^{-1}).

Type of PVS	$\mathbf{T_G}$ (°C)	$\mathbf{\Delta Cp}$ (J g $°C^{-1}$)
PVS1	-112.15 ± 0.38^{b}	1.22 ± 0.03^{a}
PVS2	-114.31 ± 0.38^{a}	$1.12 \pm 0.04^{a,b}$
PVS2 mod	-109.42 ± 0.11^{c}	1.07 ± 0.04^{b}
PVS3	-89.85 ± 0.29^{d}	0.87 ± 0.01^{b}
PVS3 mod	-86.84 ± 0.65^{e}	0.83 ± 0.01^{b}

Average values and standard deviation for at least four repeats. Means of $\mathbf{T_G}$ and $\mathbf{\Delta Cp}$, in a column, with the same letter are not significantly different according to the Duncan's Multiple Range Test at alpha = 0.05.

Table 3. Crystallization parameters (mean ± standard deviation) of garlic shoot tips after treatment with PVS3 for 2 h. Onset of the melting endotherm and crystallinity as crystallization percentage, both measured in the warming scan. Cooling scans were performed at 10ºC min^{-1} and warming scans at different rates.

Warming rate (°C min^{-1})	Onset (°C)	Crystallized (%)
5	-19.87 ± 9.40	1.96 ± 2.51
10	-16.49 ± 3.73	1.21 ± 0.25
20	-14.61 ± 0.62	0.37 ± 0.51