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Plant vitrification solution 2 lowers water content and alters freezing behavior in shoot tips during cryoprotection ☆

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Abstract

Plant shoot tips do not survive exposure to liquid nitrogen temperatures without cryoprotective treatments. Some cryoprotectant solutions, such as plant vitrification solution 2 (PVS2), dehydrate cells and decrease lethal ice formation, but the extent of dehydration and the effect on water freezing properties are not known. We examined the effect of a PVS2 cryoprotection protocol on the water content and phase behavior of mint and garlic shoot tips using differential scanning calorimetry. The temperature and enthalpy of water melting transitions in unprotected and recovering shoot tips were comparable to dilute aqueous solutions. Exposure to PVS2 changed the behavior of water in shoot tips: enthalpy of melting transitions decreased to about 40 J g H_2O^{-1} (compared to 333 J g H_2O^{-1} for pure H_2O), amount of unfrozen water increased to $\sim$ 0.7 g H_2O g dry mass^{-1} (compared to $\sim$0.4 g H_2O g dry mass^{-1} for unprotected shoot tips), and a glass transition (T_g) at −115 °C was apparent. Evaporative drying at room temperature was slower in PVS2-treated shoot tips compared to shoot tips receiving no cryoprotection treatments. We quantified the extent that ethylene glycol and dimethyl sulfoxide components permeate into shoot tips and replace some of the water. Since T_g in PVS2-treated shoot tips occurs at −115 °C, mechanisms other than glass formation prevent freezing at temperatures between 0 and −115 °C. Protection is likely a result of controlled dehydration or altered thermal properties of intracellular water. A comparison of thermodynamic measurements for cryoprotection solutions in diverse plant systems will identify efficacy among cryopreservation protocols.
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Keywords: Cryopreservation; *Allium sativum*; Garlic; *Mentha*; Mint; Shoot tip; Differential scanning calorimetry

Prevention of intracellular ice formation and growth during cooling, storage, and thawing is critical for successful cryopreservation. This is most simply accomplished by removing water that would otherwise form lethal ice crystals within cells during cooling to liquid nitrogen (LN) temperatures. Many cryopreservation protocols reduce water content either by drying desiccation tolerant tissues or by treating shoot tips with concentrated solutions which osmotically remove water from cells. Successful cryopreservation protocols balance cell water content so that both freezing injury and desiccation damage are minimized

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[15,27,36,46]. Drying cells, either by exposure to air, concentrated solutions or freeze dehydration, does not provide adequate protection from cryogenic temperatures for most non-hardy cell types. Bathing cells in solutions containing specific molecules provided a major breakthrough in modern cryobiology (e.g., [16,34]) and the discovery that some cryoprotectants are naturally produced by cold-hardy plants during winter [21] provided clear evidence that cryoprotection requires more than just adjusting water content.

Since the discovery that cells treated with specific cryoprotecting solutions survive exposure to cryogenic temperatures, variations on solution composition have been developed for plant cells [24,33,37,42,43]. These solutions are called "vitrification" or "vitrifying" solutions to emphasize their role in preventing intracellular ice by promoting glass formation in the aqueous domain of cells [12,16,35,37]. Recent concepts of aqueous glasses describe the complexity of temperature responses in these supercooled, super-viscous solutions (e.g., [1,51] and references therein). Glass transitions occur at about −115 °C in PVS2, the cryoprotectant solution most commonly used for plants [37]. Despite their name and routine usage, the mechanism by which vitrification solutions protect cells from damage is poorly understood [20]. They may function as elaborate desiccants and decrease the amount of water that is available to form lethal ice crystals [38]. Alternatively, they may stabilize cell structures during desiccation and cooling as described by literature on glassy states in dry biological systems [6,11]. Finally, vitrification agents may structure water remaining in cells so that it is less likely to freeze [54]. Cryoprotectant solutions, especially those containing glycerol, are toxic to cells and prolonged exposure can be problematic [14,17,50].

Thermal analyses of plant materials using differential scanning calorimetry (DSC) demonstrate a strong relationship between the presence of water freezing or melting transitions and damage at subfreezing temperatures [4,12,13,23,25,26,39,44–47,52]. Evidence of first order water transitions (i.e., freezing and melting) are observed in seeds (e.g., [44–46,52,18]), pollen [8], winter-hardy buds [47], naked shoot tips of olive [25], and shoot tips encapsulated in calcium alginate beads [4,13,25,39] that contain more than 0.25–0.4 g H_2O g dm^{-1} (dm; dry mass). Plant organs that survive LN temperatures usually contain this amount of water or less (e.g., [12,46,52]), though the window of acceptable water contents can be widened by increasing cooling rate to over 100 °C s^{-1} [52,53].

Water at water contents less than 0.25 g H_2O g dm^{-1} is often referred to as 'unfreezable' or 'unfrozen' to indicate that motional restrictions are too great to allow the molecular reorganizations necessary for crystallization within a practical time frame (the cooling rate in a typical DSC experiment is usually 1–20 °C min^{-1}). With such restricted mobility, water at these low water contents will vitrify with further cooling, if it has not already vitrified at room temperature, and second order transitions, representing glass formation, may be detected. Second order transitions (heat capacity changes) in seeds (e.g., [51]) are small and broad compared to those observed for simpler solutions and alginate beads (e.g., [5,25]). DSC measurements of water in biological materials may be confounded by the presence of interfering signals from lipids and by drying, cooling and warming protocols (e.g., [8,13,39,51]). Devitrification and recrystallization, observed as an exothermic event in warming DSC scans, are sometimes evident in materials containing 0.2–0.5 g H_2O g dm^{-1} [13,45] or hydrated materials treated with cryoprotectants [4,32,37], and portend a risk of damaging ice formation if cryoexposed materials are warmed as slowly as they are in DSC experiments [27,35,37]. Recrystallization does not reflect the stability of the glass per se [4,13,39], but rather the mobility within the fluid non-vitrified material. In addition to faster warming, recrystallization can be lessened by increased drying [4,7,13,45] or longer incubation in cryoprotectant solutions [23], though both procedures can damage cells in other ways.

An evaluation of the first and second order transitions and water contents of cryoprotected shoot tips will reveal potential mechanisms by which PVS2 is effective. Comparisons of the thermal properties of treated biological materials and cryoprotectant solutions provide valuable insights towards understanding the mechanism of cryoprotection [3,5,15]. We have used DSC to measure the size and temperature of first (exothermic and endothermic events) and second order transitions (glass transitions) within treated shoot tips of garlic and mint at each step of a cryopreservation procedure that uses PVS2 as the cryoprotectant. Bathing garlic shoot tips in cryoprotectant solutions reduces the size and sometimes the temperature of first order transitions [23], but it is unclear whether freezing is reduced because the cells dried out in response to the cryoprotectants or because the cryoprotectants altered the