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THERMAL ANALYSIS OF GARLIC SHOOT TIPS DURING A VITRIFICATION PROCEDURE

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Abstract

The thermal behavior of garlic shoot tips was analyzed during the course of a vitrification protocol using the PVS3 vitrification solution. The size of shoot tips did not significantly influence the thermal behavior of garlic shoot tips. Though there was no significance, endothermal enthalpy from melting of crystalline ice increased as preculture duration increased to 6 days. Preculture on medium with 0.5 M sucrose significantly lowered exo- and endothermal enthalpies of dehydration-control shoot tips. By contrast, after dehydration with PVS3 solution, the concentration of sucrose in preculture medium had no significant effect on the value of enthalpies. A big thermal event was observed in garlic shoot tips air-dried for 1-3 h before dehydration. Both vitrification solution and dehydration duration significantly ($P < 0.0001$) influenced exo- and endothermal enthalpies. After dehydration with PVS1, PVS2, Fahy or Steponkus solutions for 120 min, only a small peak was detected in some shoot tips, but recovery of cryopreserved shoot tips was low. Dehydration duration with PVS3 solution significantly ($P < 0.0001$) influenced exo- and endothermal enthalpies and onset temperatures during cooling and warming. After dehydration for 150 and 180 min with PVS3 vitrification solution, no crystallization was observed during cooling and warming in most replicates, and recovery of cryopreserved shoot tips was highest (> 80 %). There was a significant ($P < 0.001$) negative correlation between moisture content of shoot tips and concentration of sucrose and glycerol, and regeneration of cryopreserved shoot tips. By contrast, there was a significant ($P < 0.001$) positive correlation between MC and enthalpy of ice melting, and onset temperature of crystallization. Overall, the results of the analysis of the thermal behavior of garlic shoot tips coincide very well with their recovery after cryopreservation and provide a very useful tool for the establishment and optimization of cryopreservation protocols.

Keywords: *Allium sativum* L., DSC, PVS3, vitrification

INTRODUCTION

In our laboratory, garlic is used as a model to study the various parameters involved in the establishment of cryopreservation protocols for shoot tips of bulbaceous crops. We focused

our first studies on the optimization of the successive steps of the cryopreservation protocol. Our results showed that recovery of cryopreserved shoot tips was highest using smaller explants excised from larger bulbs and bulbils, which had been stored for 3-6 months after harvest (2). Optimal treatment conditions included preculture on medium with 0.3-0.5 M sucrose for 1-2 days followed by dehydration with the PVS3 (19) solution for 150-180 min, rapid freezing and rapid rewarming in a water-bath at 37°C (12). The growth regulator content of the recovery medium did not influence percent regeneration. However, the fresh weight of shoot tips cultured on medium containing 0.3 mg/l zeatin and gibberellic acid was significantly higher than on other media. This optimized cryopreservation protocol was applied to ten different garlic varieties, with regeneration percentages ranging between 72.2 and 95.0%.

In a second set of experiments, we focused our attention on a subject little studied in cryopreservation research, *i.e.* the evolution of cryoprotectant concentration in explants during a vitrification procedure. We measured the evolution in garlic shoot tips of DMSO concentration during vitrification with the PVS2 (22) solution (13), and that of sucrose and glycerol during vitrification with the PVS3 solution and showed that influx and efflux of cryoprotectants in/out of garlic shoot tips were very rapid (14).

A very positive development in cryopreservation research is that an increasing number of researchers systematically use analytical tools (e.g. histo-cytology, differential scanning calorimetry, biochemical studies) to establish cryopreservation protocols, which allows the development of a scientific basis to the success/failure of experimental treatments (5,9,10). Analytical tools can be used to profile the injurious events, which occur when tissues are cryopreserved (5,6,7). Such profiles allow the detection of those components of a cryopreservation method, which cause most damage. Usually, these studies are correlated with survival and viability testing. Once damaging events have been identified, specific measures can be taken to reduce injury and enhance survival.

In the new, vitrification-based, cryopreservation protocols, elimination of most or all freezable water from samples by means of physical or chemical dehydration before freezing is considered the key step for success (4,8,9). It is thus of paramount importance to be able to measures the moisture content of samples throughout a cryopreservation protocol. Among the analytical tools available, Differential Scanning Calorimetry (DSC), which very accurately measure the nature and intensity of thermal events during a freeze-thaw cycle, has been used with a range of plant materials to correlate survival of frozen samples with these thermal events. An increase in survival is systematically correlated with a decrease in the intensity of ice crystallization/ice melting events and optimal survival is usually reached when only vitrification events are recorded (1,3,5,8,16,21). A DSC study of alginate beads used in encapsulation-dehydration techniques of cryopreservation has shown that many factors, including sample pre-treatment, dehydration, etc are important in optimizing protocols for vitrification (5).

In this paper, garlic shoot tips were cryopreserved using a vitrification protocol employing the PVS3 solution. We studied the influence of various parameters of the protocol on the nature and intensity of the thermal events recorded by DSC in garlic shoot tips during cooling and warming.

MATERIALS AND METHODS

Plant material

The Korean garlic cv. Eiusung was used in this study. Bulb scales were planted in Suwon, Korea in September 2001 in plastic greenhouses with mulching provided by black plastic film. Bulbs were harvested at the end of June 2002, left to dry in the plastic greenhouse for 3

weeks and then stored in a cold room at 0-2°C with 65-70 % relative humidity (RH) for 2 to 7 months until sampling. Experiments started in early September 2002, when dormancy had been broken in most of the clove shoot tips, and finished by mid-February 2003.

Extraction and disinfection of shoot tips

Shoot tips were extracted from garlic cloves using borers with a diameter between 1.5 and 4.5 mm. The basal plates were cut with a scalpel (No. 11 blade) until they were 0.7 to 1.0 mm thick. The upper parts of the shoot tips were then trimmed until explants were 3.0 to 3.5 mm long. The explants employed for freezing consisted of the meristematic dome, the surrounding leaf primordia and a basal part. For surface sterilization, shoot tips were washed twice in 200 ml tap water with 2-3 drops (2 ml) of commercial detergent for 2-3 min each and then immersed in 80% ethanol for 1 min. After rinsing with distilled water, they were placed in a 0.5% sodium hypochlorite solution for 12 min with continuous shaking, and then rinsed at least four times with sterilized water under the laminar airflow cabinet.

Vitrification procedure

After surface sterilization, shoot tips were inoculated on solid hormone free Murashige and Skoog (MS) medium (18) containing 3 % sucrose and 2.5 g/l phytagel (Sigma Co.) and precultured on this medium for 3 days at 10°C, under a 16 h light/8 h dark photoperiod, with a light intensity of 60 µmol/m^2/s. For dehydration, shoot tips were placed in PVS3 solution (50 % sucrose (w/v) + 50 % glycerol (w/v) in MS medium) (30 shoot tips in 30 ml PVS3 solution) under constant shaking (90 rpm) for 150 min at 23°C.

In some experiments, shoot tips were cryopreserved in parallel with DSC measurements. The procedures employed for freezing, thawing, unloading and regrowth of shoot tips were those described by Kim *et al*. (12).

Recovery assessment and moisture content

Recovery was evaluated 30 days after cryopreservation by counting the number of apices, which had developed leaves. In all experiments, a minimum of 20 apices was used per experimental condition and experiments were replicated 3-5 times.

The moisture content (% MC, fresh weight basis) of shoot tips was measured at various stages of the cryopreservation protocol. Dry weight was measured using 20-25 shoot tips after 48 h drying in an oven at 105°C.

Thermal analysis

The thermal analysis system used in this investigation was a DSC822 (TA8000 Mettler-Toledo, GMbh, Switzerland) incorporating a heat flux module, liquid nitrogen cooling system and an Epson TAS811 workstation employing STAR^e Software.

For DSC analysis, shoot tips were sampled randomly from the different treatments tested, weighed on an analytical balance (precision ± 1µg) and placed individually in 100 µl aluminum pans for scanning. The average individual fresh weight of shoot tips was 13~20 mg after preculture and 8~18 mg after dehydration with the PVS3 solution. Scans involved cooling with a scanning rate of 10°C/min from 25 to -65°C, followed by isothermal hold at -65°C for 2 min to allow sample equilibration. The samples were then heated to 25°C using a linear scanning rate of 10°C/min. Analyses were performed using an average of four shoot tips for each experimental condition, even though in some cases 3 to 9 samples were used. The thermograms obtained from each run were analyzed using the DSC evaluation software, to obtain onset temperatures of crystallization and of ice melting, and the midpoint temperature of the glass transition (*T*g), and enthalpies. Enthalpies were determined from the area of the peaks above or below the interpolated baseline.

The effect of the following parameters on thermal behavior of shoot tips was investigated:

- Size of shoot tips, which varied from 1.5 to 4.5 mm in diameter and from 3.0 to 3.5 mm in length. Shoot tips were extracted from cloves with borers of the corresponding diameter.
- Preculture duration for 1, 3 or 6 days at 10 or 23°C.
- Concentration of sucrose in preculture medium (no preculture, 0.1, 0.3, and 0.5 M), followed by dehydration with PVS3 for 150 min.
- Air-drying for 0-3 h under the laminar airflow bench before dehydration with PVS3 for 150 min, or for 1 h after dehydration with PVS3 for 150 min.
- Dehydration with different vitrification solutions (Table 1) for 40, 80 or 120 min.
- Duration of dehydration with PVS3 solution, from 0 to 180 min.
- Preconditioning treatments including preculture with sucrose 0.1 M (PC) or 0.5 M (PCS) for 2 days at 10°C, cold acclimation for 10 days at 5°C (CA), loading with 0.4 M sucrose + 2 M glycerol solution for 30 min (LD), and dehydration with PVS3 for 150 min (DH).

Table 1. Composition of the various vitrification solutions used in garlic shoot tip cryopreservation experiments.

Solution	Composition	Reference
PVS1	Glycerol 22 % + propylene glycol 13 % + EG 13 % + DMSO 6 % in MS with 0.4 M sucrose	Uragami et al. (24)
PVS2	Glycerol 30 % + DMSO 15 % + EG 15 % in MS with 0.4 M sucrose	Sakai et al. (22)
PVS3	Glycerol 50 % + sucrose 50 % in MS (without 0.4 M sucrose) Glycerol 35 % + ethylene glycol 20 % + 0.6 M sucrose in MS	Nishizawa et al. (19)
PVS4	EG 50 % + sorbitol 15 % + BSA 6 % (in MS with 0.4 M sucrose)	Matsumoto (17)
Steponkus	EG 35 % + DMSO 1 M + PEG 8000 10 % (in MS with 0.4 M sucrose)	Langis et al. (15)
Towill	DMSO 20 % + formamide 20 % + propylene glycol 15 %	Towill (23)
Fahy		Fahy et al. (11)

Measurement of sucrose and glycerol concentration

The sucrose and glycerol concentration of shoot tips was measured by high-performance liquid chromatography (HPLC), following the method described by Kim *et al*. (14).

Statistical analysis

Mean values ± standard deviations of DSC measurements were calculated from 3-9 replicates. Results were analyzed using the least significance deviation (LSD) or Duncan's multiple range test (DMRT), with the SAS 8.1 software.

RESULTS

During the cooling and warming phases, exo- and endothermal events were observed in shoot tips dehydrated for 90 and 150 min with PVS3 solution regardless of their size, indicating the occurrence of ice crystal formation during cooling and ice melting during warming (Table 2). Even though the differences observed in the various parameters studied were not significant between apices of different sizes, larger apices (4.5 x 3.5 mm) displayed

higher enthalpies than smaller ones after both PVS3 dehydration durations tested. Recovery of larger apices was significantly lower than that of smaller ones.

Table 2. Thermal characteristics of garlic shoot tips, dehydrated with PVS3 for 90 or 150 min, during cooling and warming as a function of shoot tip size and recovery (%) after freezing.

Shoot tip size[†] and dehydration duration (min)[‡]		Cooling		Warming		Recovery
		Enthalpy (J/g FW)	Onset (°C)	Enthalpy (J/g FW)	Onset (°C)	(%)
1.5 x 3.0	90	3.7 ± 4.0 [a]	-31.8 ± 7.0 [ab]	-4.7 ± 5.1 [ab]	-29.2 ± 13.7 [a]	-
	150	0.3 ± 0.5 [a]	-21.5 ± 6.4 [a]	-0.2 ± 0.4 [a]	-47.1 ± 0.0 [a]	73.9 ± 13.7 [a]
3.0 x 3.0	90	1.3 ± 2.3 [a]	-42.9 ± 5.4 [b]	-2.8 ± 2.9 [ab]	-37.2 ± 0.9 [a]	-
	150	0.3 ± 0.4 [a]	-45.3 ± 7.3 [b]	-0.4 ± 0.6 [a]	-28.1 ± 10.0 [a]	90.7 ± 3.9 [a]
4.5 x 3.5	90	7.6 ± 11.5 [a]	-36.8 ± 5.7 [b]	-9.6 ± 11.4 [b]	-36.3 ± 3.9 [a]	-
	150	3.6 ± 7.3 [a]	-31.8 ± 0.0 [ab]	-4.9 ± 8.0 [ab]	-40.3 ± 0.0 [a]	19.6 ± 17.0 [b]
Pr>F		ns	ns	ns	ns	*

[†]Diameter x height (mm, including a 0.7-1.0 mm thick basal plate). [‡]Shoot tips were dehydrated in PVS3 solution for 90 or 150 min at 23°C before DSC analysis. -: not measured. ns: non significant at the 95 % significance level based on LSD.

Even though the temperature and duration of preculture of shoot tips had no significant effect on enthalpy and onset and peak temperatures, except enthalpy during warming, enthalpy increased as preculture duration increased (Table 3). The largest thermal events were observed during warming in shoot tips precultured for 6 days at 23°C (-11.6 J/g FW) or 10°C (-4.1 J/g FW). Recovery of cryopreserved shoot tips was lower at both preculture temperatures for the longest durations tested.

Table 3. Thermal characteristics of garlic shoot tips, dehydrated with PVS3 for 150 min, during cooling and warming as a function of preculture temperature and duration, and recovery (%) after freezing.

Preculture temperature and duration (days)		Cooling		Warming		Recovery
		Enthalpy (J/g FW)	Onset (°C)	Enthalpy (J/g FW)	Onset (°C)	(%)
10°C	1	1.6 ± 3.2 [b]	-29.0 ± 0.0 [a]	-1.5 ± 3.3 [a]	-11.2 ± 14.1 [a]	66.7 ± 10.4 [ab]
	3	0.3 ± 0.5 [b]	-39.0 ± 0.0 [a]	-0.2 ± 0.3 [a]	-16.6 ± 0.0 [ab]	82.3 ± 9.0 [a]
	6	3.0 ± 4.1 [ab]	-32.9 ± 5.6 [a]	-4.1 ± 5.4 [ab]	-22.8 ± 12.0 [ab]	64.8 ± 15.7 [b]
23°C	1	0.4 ± 0.6 [b]	-39.9 ± 7.9 [a]	-0.6 ± 0.4 [a]	-20.9 ± 16.4 [ab]	80.1 ± 15.6 [ab]
	3	1.4 ± 2.5 [b]	-38.9 ± 6.6 [a]	-2.0 ± 3.1 [a]	-26.8 ± 3.3 [ab]	70.1 ± 12.0 [ab]
	6	8.2 ± 7.6 [a]	-35.4 ± 4.0 [a]	-11.6 ± 9.9 [b]	-35.4 ± 2.7 [b]	15.9 ± 5.9 [c]
Pr>F		ns	ns	*	ns	**

Shoot tips (3 mm in diameter with a 0.7-1.0 mm thick basal plate) were precultured on medium with 0.1 M sucrose, then dehydrated in PVS3 solution for 150 min at 23 °C before DSC analysis. Figures in columns followed by the same letter are not different at the 95% (*) and 99% (**) significance level of the ANOVA table based on DMRT. ns: non significant.

In shoot tips which were precultured but not treated with the PVS3 solution, the sucrose concentration of the preculture medium had a highly significant ($P < 0.0001$) effect on the intensity of the exo- and endothermal enthalpies, and significantly influenced the onset and midpoint temperatures measured during both cooling and warming (Table 4). The higher the sucrose concentration in the preculture medium, the lower the enthalpy, onset and peak

temperature measured. By contrast, the onset and midpoint temperatures of shoot tips treated with the PVS3 vitrification solution after preculture, measured during both cooling and warming, did not vary significantly depending on the sucrose concentration of preculture medium. Recovery was nil without PVS3 treatment and increased significantly with sucrose concentrations of 0.3 and 0.5 M in the preculture medium with PVS3-dehydrated shoot tips.

Table 4. Thermal characteristics of garlic shoot tips, dehydrated (DH), or not, with PVS3 for 150 min, during cooling and warming, as a function of the temperature and duration of preculture, and recovery (%) after freezing.

Sucrose concentration (M) with and without dehydration(D)	Cooling		Warming		Recovery
	Enthalpy (J/g FW)	Onset (°C)	Enthalpy (J/g FW)	Onset (°C)	(%)
PC control	225.6 ± 11.1^{b}	-9.5 ± 0.4^{a}	-207.9 ± 8.7^{c}	-2.4 ± 0.1^{a}	0^{d}
0.1 M	241.8 ± 6.2^{a}	-12.3 ± 3.9^{a}	-208.7 ± 14.5^{c}	-4.3 ± 3.0^{a}	0^{d}
0.3 M	214.1 ± 10.5^{c}	-15.2 ± 2.5^{ab}	-198.4 ± 8.1^{c}	-5.1 ± 1.9^{a}	0^{d}
0.5 M	122.7 ± 2.7^{d}	-22.2 ± 6.0^{b}	-116.9 ± 5.3^{b}	-17.5 ± 0.4^{b}	0^{d}
Pr>F	***	*	***	***	ns
PC control –DH	8.3 ± 0.5^{e}	-33.9 ± 0.3^{c}	-7.4 ± 0.6^{a}	-22.2 ± 1.5^{bc}	66.4 ± 5.7^{c}
0.1 M – DH	0.3 ± 0.3^{e}	-44.5 ± 1.2^{d}	-1.8 ± 0.5^{a}	-33.1 ± 0.9^{c}	72.2 ± 6.9^{b}
0.3 M – DH	0.4 ± 0.5^{e}	-41.0 ± 5.3^{cd}	-0.8 ± 1.4^{a}	-21.0 ± 14.4^{bc}	85.1 ± 2.3^{a}
0.5 M – DH	0.4 ± 0.8^{e}	-38.2±0.0^{cd}	-0.8 ± 0.9^{a}	-19.2 ± 10.8^{b}	87.7 ± 3.2^{a}
Pr>F	***	ns	**	ns	***
Pr>F	***	***	***	**	**

Shoot tips were precultured on medium with 0.1, 0.3 or 0.5 M sucrose, and then dehydrated in PVS3 solution for 150 min at 23°C (DH) before DSC analysis. PC control: shoot tips not submitted to any preculture treatment. Figures in columns followed by the same letter are not different at the 95% (*), 99% (**) and 99.9% (***) significance level of the ANOVA table based on DMRT. ns: non significant.

Table 5. Effect of air-drying treatment (AD) duration, of treatment with PVS3 vitrification solution (DH) and of the treatment sequence (AD-DH/DH-AD) on moisture content (MC, % FW) and thermal characteristics of garlic shoot tips during cooling and warming.

Treatment sequence[†]		Shoot tip MC (%)		Cooling		Warming		Recovery (%)
Step 1	Step 2	Step 1	Step 2	Enthalpy (J/g FW)	Onset (°C)	Enthalpy (J/g FW)	Onset (°C)	
Control	DH	84.2^{a}	33.1^{a}	0.3 ± 0.4^{b}	-46.5 ± 6.6^{c}	-1.0 ± 1.5^{ab}	-31.1 ± 7.3^{a}	88.9 ± 5.7^{a}
AD 1h	DH	72.4^{b}	32.9^{a}	5.0 ± 6.8^{a}	-32.8 ± 5.4$^{a\,b}$	-6.0 ± 7.5^{b}	-22.2 ± 8.6^{a}	48.6 ± 24.0^{bc}
AD 2h	DH	64.2^{c}	29.2^{b}	2.5 ± 2.7^{ab}	-39.3 ± 7.2^{bc}	-4.2 ± 3.3^{ab}	-24.0 ± 4.9^{a}	44.8 ± 34.6^{bc}
AD 3h	DH	58.1^{d}	27.5^{b}	1.8 ± 1.8^{ab}	-23.1 ± 13.8^{a}	-2.8 ± 2.8^{ab}	-33.5 ± 11.6^{a}	25.4 ± 27.2^{c}
DH	AD 1h	33.1^{e}	25.4^{c}	0.0 ± 0.0^{b}	-	-0.1 ± 0.3^{a}	-32.9 ± 0.0^{a}	52.6 ± 25.8^{b}
Pr >F				ns	ns	ns	ns	***

[†] Precultured shoot tips were air-dried for 0-3 h before dehydration with PVS3 solution for 150 min or air-dried for 1 h after PVS3 dehydration. Figures in columns followed by the same letter are not different at the 99.9% (***) significance level of the ANOVA table based on DMRT. ns: non significant.

Performing the air dehydration step after the PVS3 treatment resulted in a lower shoot tip MC than when air dehydration was carried out before the PVS3 treatment (Table 5). Although differences were not significant, increasing the duration of air-drying before the PVS3 treatment progressively decreased the intensity of the exo- and endothermic events measured in shoot tips. No thermal event was observed during cooling of shoot tips air-dried for 1 h following PVS3 dehydration, but a small thermal event was still measured during warming in some shoot tips. Air-drying treatment before or after dehydration with PVS3 significantly ($P < 0.001$) decreased recovery of cryopreserved shoot tips. Air-drying before PVS3 treatment significantly decreased recovery in comparison with shoot tips treated directly with the PVS3 solution. An additional 1 h air-drying period after the PVS3 treatment was also detrimental to recovery.

Table 6. Effect of vitrification solution and dehydration duration on moisture content (MC, % FW) and thermal characteristics of garlic shoot tips during cooling and warming, and recovery (%) after freezing.

Vitrification solution and dehydration duration (min)	MC (%)	Cooling		Warming		Recovery (%)
		Enthalpy (J/g FW)	Onset (°C)	Enthalpy (J/g FW)	Onset (°C)	
PVS1 40	62.2$^{c\,d}$	8.5 ± 2.7$^{c\,d}$	-34.5 ± 7.9$^{a\,b}$	-12.8 ± 2.5^{bc}	-48.1 ± 3.5^{d}	1.7 ± 2.9^{de}
80	61.1$^{c\,d}$	0.2 ± 0.4^{d}	-52.6 ± 0.0^{b}	-0.3 ± 0.4^{a}	-45.3 ± 0.0$^{c\,d}$	-
120	60.8$^{c\,d}$	0.1 ± 0.2^{d}	-46.2 ± 0.0^{b}	-0.1 ± 0.2^{a}	-46.0 ± 0.0$^{c\,d}$	10.8 ± 6.1$^{c\,de}$
PVS2 40	61.2$^{c\,d}$	3.6 ± 6.9^{d}	-37.6 ± 9.6^{b}	-4.2 ± 7.7$^{a\,b}$	-40.2 ± 3.6^{bcd}	26.5 ± 15.0^{bc}
80	57.0^{e}	0.3 ± 0.4^{d}	-36.1 ± 0.0^{b}	-0.1 ± 0.1^{a}	-10.8 ± 0.0^{a}	-
120	56.5^{e}	0.1 ± 0.2^{d}	-42.6 ± 0.0^{b}	-0.0 ± 0.1^{a}	-26.8 ± 0.0$^{a\,bc}$	22.2 ± 5.8$^{bc\,de}$
PVS3 40	50.8^{f}	7.0 ± 6.5$^{c\,d}$	-33.5 ± 5.5$^{a\,b}$	-9.5 ± 6.8$^{a\,b}$	-39.6 ± 1.8^{bcd}	27.1 ± 21.7^{bc}
80	42.0^{g}	2.7 ± 3.6^{d}	-15.9 ± 15.3^{a}	-3.0 ± 3.9^{a}	-40.1 ± 1.5^{bcd}	-
120	35.6^{h}	1.7 ± 2.2^{d}	-35.4 ± 3.8^{b}	-2.2 ± 2.9^{a}	-37.9 ± 15.5^{bcd}	80.4 ± 11.9^{a}
PVS4 40	61.3$^{c\,d}$	18.4 ± 10.1^{ab}	-35.8 ± 7.4^{b}	-26.2 ± 12.4^{d}	-47.9 ± 2.1$^{c\,d}$	22.9 ± 20.2^{bcde}
80	57.5^{e}	0.3 ± 0.4^{d}	-51.6 ± 4.5^{b}	-9.9 ± 2.6$^{a\,b}$	-43.7 ± 0.9$^{bc\,d}$	-
120	56.6^{e}	0.4 ± 0.8^{d}	-33.9 ± 12.1$^{a\,b}$	-2.7 ± 3.0^{a}	-43.7 ± 1.3$^{bc\,d}$	25.3 ± 22.1^{bcd}
Steponkus 40	62.9^{c}	12.7 ± 5.7^{bc}	-32.8 ± 2.9$^{a\,b}$	-13.8 ± 5.9^{bc}	-46.4 ± 15.0$^{c\,d}$	22.7 ± 17.7^{bcde}
80	58.1^{e}	0.1 ± 0.3^{d}	-41.1 ± 0.0^{b}	-0.1 ± 0.1^{a}	-23 .6 ± 0.0$^{a\,b}$	-
120	58.0^{e}	0.2 ± 0.3^{d}	-41.9 ± 0.0^{b}	-0.4 ± 0.7^{a}	-41.1 ± 0.0$^{bc\,d}$	5.0 ± 8.7$^{c\,de}$
Towill 40	65.0^{b}	22.7 ± 7.7^{a}	-36.9 ± 3.7^{b}	-26.1 ± 3.3^{d}	-47.2 ± 5.1$^{c\,d}$	39.2 ± 34.3^{b}
80	62.7^{c}	18.6 ± 15.4^{ab}	-41.0 ± 7.0^{b}	-21.6 ± 11.5^{cd}	-47.2 ± 3.0$^{c\,d}$	-
120	60.3^{d}	1.5 ± 2.3^{d}	-48.2 ± 2.3^{b}	-9.2 ± 10.1$^{a\,b}$	-48.7 ± 0.5^{d}	8.6 ± 3.5$^{c\,de}$
Fahy 40	70.3^{a}	9.6 ± 5.2$^{c\,d}$	-37.6 ± 11.4^{b}	-13.8 ± 10.2^{bc}	-44.5 ± 12.4$^{c\,d}$	10.8 ± 10.1$^{c\,de}$
80	66.9^{b}	0.4 ± 0.8^{d}	-37.6 ± 0.0^{b}	-0.3 ± 0.6^{a}	-14.3 ± 0.0^{a}	-
120	66.7^{b}	0.3 ± 0.5^{d}	-41.0 ± 0.0^{b}	-0.1 ± 0.1^{a}	-12.2 ± 0.0^{a}	0.0 ± 0.0^{e}
Pr > F	***	***	*	***	**	***
Solution		**	ns	**	**	***
Duration		**	ns	**	**	ns
Sol.*dur.		*	*	ns	ns	***

Shoot tips were dehydrated at 23°C for 40, 80 or 120 min with various vitrification solutions before DSC analysis. Figures in columns followed by the same letter are not different at the 95% (*), 99% (**) and 99.9% (***) significance level of the ANOVA table based on DMRT. ns: non significant. -: not measured.

The MC of shoot tips dehydrated with the PVS1, PVS2, PVS4, Steponkus, Towill and Fahy solutions varied within similar ranges, from 61.2-70.3% after 40 min to 56.5-66.7% after 120 min (Table 6). Dehydration of shoot tips with the PVS3 solution led to much lower MCs, from 50.8% after 40 min to 35.6% after 120 min and recovery was maximal for shoot tips treated for 120 min with the PVS3 solution. Both vitrification solution and dehydration duration significantly ($P < 0.0001$) influenced the intensity of the exo- and endothermal enthalpies. Shoot tips treated for 40 min with all vitrification solutions tested showed crystallization and ice melting peaks during both cooling and warming. After dehydration for 120 min, relatively large peaks were still observed during cooling and warming of shoot tips treated with PVS3, PVS4 and Towill solution. By contrast, after dehydration for 120 min with PVS1, PVS2, Steponkus and Fahy solutions, small crystallization/ice melting peaks were still observed during cooling/warming in one or two of the four shoot tips studied, whereas only glass transitions were recorded in the other samples. Cytotoxicity is thus the cause of the relatively low recovery of shoot tips dehydrated with these solutions.

The duration of dehydration with PVS3 solution significantly influenced the intensity of the exo- and endotherms, as well as the onset and midpoint temperatures measured during cooling and warming of shoot tips (Table 7). As dehydration duration increased from zero to 60 min, the enthalpy decreased from –141.3 J/g FW to –11.5 J/g FW and from 146.6 J/g FW to 11.2 J/g FW during warming and cooling, respectively and after 180 min, it reached -0.3 J/g FW and 0.0 J/g FW for warming and cooling, respectively. Recovery was nil up to 30 min of PVS3 treatment and increased progressively to reach 81.4 - 92.4% for treatment durations between 120 and 180 min.

Table 7. Moisture content (% FW) and thermal characteristics of garlic shoot tips during cooling and warming as a function of the duration of dehydration with PVS3 solution, and recovery (%) after freezing.

Dehydration (min)	MC (%)	Cooling		Warming		Recovery
		Enthalpy (J/g FW)	Onset (°C)	Enthalpy (J/g FW)	Onset (°C)	(%)
0	83.1^{a}	146.6 ± 4.2^{a}	-10.9 ± 2.0^{a}	-141.3 ± 11.8^{f}	-0.2 ± 1.6^{a}	0.0^{d}
10	61.4^{b}	107.5 ± 24.8^{b}	-23.1 ± 3.6^{b}	-100.1 ± 22.6^{e}	-24.9 ± 8.7^{b}	0.0^{d}
20	53.8^{c}	70.1 ± 23.0^{c}	-23.8 ± 6.1^{b}	-67.5 ± 20.7^{d}	-24.0 ± 8.7^{b}	0.0^{d}
30	46.7^{d}	37.5 ± 21.7^{d}	-27.8 ± 3.5^{bc}	-36.0 ± 17.4^{c}	-25.6 ± 11.5^{b}	0.0^{d}
45	43.5^{e}	16.9 ± 7.7^{e}	-35.4 ± 8.5^{cd}	-17.6 ± 6.3^{b}	-36.1 ± 4.1^{b}	-
60	40.6^{f}	11.2 ± 9.6^{e}	-30.5 ± 7.1^{bcd}	-11.5 ± 9.9^{ab}	-30.3 ± 8.9^{b}	21.4 ± 5.1^{c}
90	35.0^{g}	1.9 ± 2.4^{e}	-37.1 ± 7.1^{d}	-2.5 ± 2.8^{ab}	-30.3 ± 12.0^{b}	47.2 ± 3.9^{b}
120	32.9^{h}	1.2 ± 1.4^{e}	-36.3 ± 3.6^{cd}	-1.6 ± 1.9^{ab}	-37.3 ± 17.8^{b}	81.4 ± 3.5^{a}
150	31.1^{hi}	0.3 ± 0.4^{e}	-46.5 ± 6.6^{e}	-1.0 ± 1.5^{ab}	-31.1 ± 7.3^{b}	92.0 ± 2.4^{a}
180	29.7^{i}	0.0 ± 0.0^{e}	-55.7 ± 0.0^{f}	-0.3 ± 0.4^{a}	-36.4 ± 0.0^{b}	92.4 ± 6.9^{a}
Pr > F	***	***	***	***	**	***

Figures in columns followed by the same letter are not different at the 99% (**) and 99.9 % (***) significance level of the ANOVA table based on DMRT. -: not measured.

Shoot tips cryopreserved without PVS3 treatment did not show any recovery (Table 8). They also displayed very high enthalpies and onset temperatures in comparison with the other conditions applied. The highest recovery percentages after cryopreservation were achieved after treatment combinations including preculture on medium with 0.5 M sucrose followed by PVS3 dehydration (96.7% recovery), cold acclimation followed by PVS3 dehydration (91.1% recovery), and preculture on medium with 0.1 M sucrose followed by loading with sucrose

and glycerol and PVS3 dehydration (90.1% recovery). Under these conditions, the enthalpy and onset temperatures during cooling and warming were significantly lower than without PVS3 treatment. By contrast, no significant difference was noted between experimental conditions for the onset temperature recorded during warming.

Table 8. Effect of various treatment combinations (preculture, cold acclimation, loading, dehydration with PVS3 solution) on the recovery of cryopreserved garlic shoot tips (%) and thermal characteristics of shoot tips during cooling and warming, and recovery (%) after freezing.

Treatment	Cooling		Warming		Recovery (%)
	Enthalpy (J/g FW)	Onset (°C)	Enthalpy (J/g FW)	Onset (°C)	
Fresh	133.3 ± 14.8^{b}	-10.7 ± 1.3^{a}	-137.6 ± 14.3^{b}	-18.3 ± 3.2^{a}	0.0^{d}
PC	146.6 ± 4.2^{a}	-10.9 ± 2.0^{a}	-141.3 ± 11.8^{b}	-21.7 ± 1.6^{a}	0.0^{d}
DH	0.8 ± 0.7^{c}	-34.9 ± 10.4^{b}	-1.2 ± 1.0^{a}	-25.9 ± 8.1^{a}	76.4 ± 6.1^{c}
PC-DH	0.5 ± 1.6^{c}	-44.2 ± 8.2^{b}	-1.1 ± 2.0^{a}	-34.6 ± 2.6^{a}	82.3 ± 2.4^{c}
CA-DH	0.03 ± 0.0^{c}	-52.0 ± 0.0^{b}	-0.4 ± 0.5^{a}	-23.1 ± 20.1^{a}	91.1 ± 5.2^{ab}
PCS-DH	0.03 ± 0.0^{c}	-45.0 ± 0.0^{b}	-0.1 ± 0.3^{a}	-28.2 ± 0.0^{a}	96.7 ± 3.0^{a}
PC-LD-DH	0.1 ± 0.3^{c}	-36.9 ± 0.0^{b}	-1.5 ± 3.3^{a}	-39.3 ± 0.0^{a}	90.1 ± 2.1^{b}
Pr > F	***	***	***	ns	***

Fresh: no treatment; PC, preculture with 0.1 M sucrose for 2 days at 10°C; DH, dehydration with PVS3 solution for 150 min; CA, cold acclimation for 10 days at 5°C on medium with 0.1M sucrose; PCS, preculture with 0.5 M sucrose for 2 days at 10°C; LD, loading with 0.4 M sucrose + 2 M glycerol solution for 30 min. Figures in columns followed by the same letter are not different at the 99% (**) and 99.9% (***) significance level of the ANOVA table based on DMRT. ns: non significant.

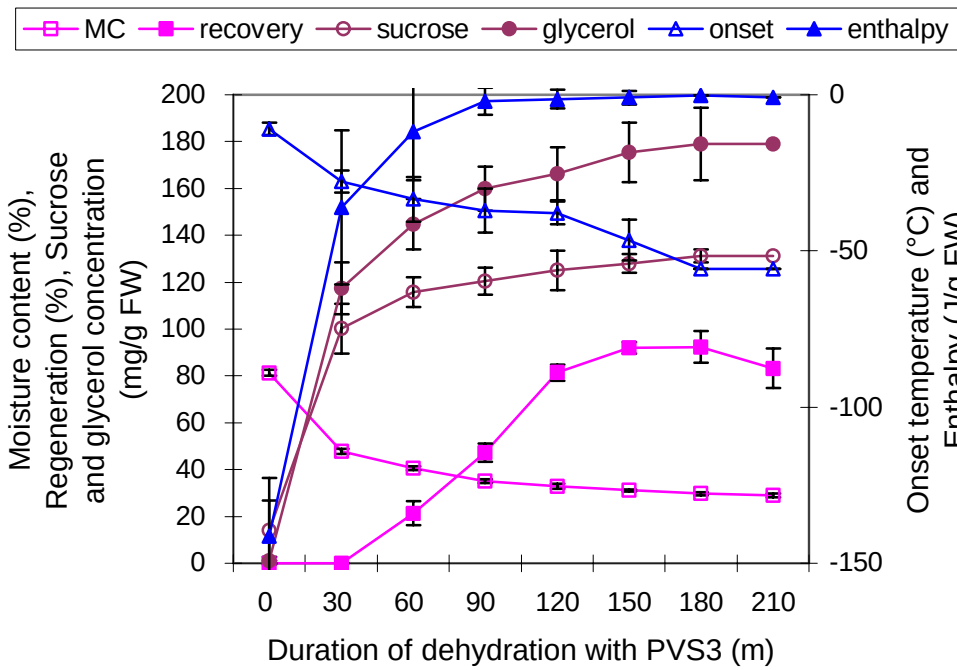

Figure 1. Thermal characteristics (onset temperature of crystallization (°C), enthalpy of ice melting (J/g FW)), concentration of sucrose and glycerol in shoot tips (mg/g FW) and recovery of cryopreserved garlic shoot tips (%) as a function of shoot tip MC (% FW) during dehydration with PVS3 solution for 0-210 min.

There was a highly significant ($P < 0.001$) negative correlation between shoot tip MC and sucrose and glycerol concentration (coefficients of correlation -0.994 and -0.998, respectively), and regeneration of cryopreserved shoot tips (coefficient of correlation -0.806) (Fig. 1). There was a highly significant ($P < 0.001$) positive correlation between shoot tip MC and enthalpy of ice melting (coefficient of correlation 0.987), and onset temperature of crystallization (coefficient of correlation 0.896).

After dehydration with the PVS3 solution for 90 min, MC decreased from 83.1% to 35%. During this period, both endothermal enthalpy and exothermal onset temperature linearly decreased from –141.3 to –2.5 J/g FW and from -10.9 to -37.1°C, respectively. From this point onwards, regeneration of cryopreserved shoot tips drastically increased. Dehydration for 90-210 min did not significantly ($P < 0.05$) increase enthalpies, but did drastically decrease onset temperatures, whereas shoot tip MC remained stable. Regeneration of cryopreserved shoot tips was nil for MCs equal to or higher than 47.7%, then increased progressively to reach a maximum (92.0%) at 31.0% MC and then decreased again for lower MCs.

Sucrose and glycerol concentration increased rapidly during the first 30 min of PVS3 treatment, corresponding to a rapid decrease in shoot tip MC, then progressively more slowly until 210 min PVS3 treatment. Shoot tip MC decreased only slightly during the same period.

DISCUSSION

Changes measured in the overall thermal behavior of cryopreserved garlic shoot tips coincide very well with changes observed in their recovery percentages, as a function of the parameters studied, including shoot tip size, preculture temperature and duration, air-drying treatment, sucrose concentration in preculture medium, vitrification solution and dehydration duration (2,12). In some vitrification solutions, *i.e.* PVS1, PVS2, Steponkus, Fahy solution, cytotoxicity of the vitrification solution employed may influence the regeneration of dehydrated and cryopreserved samples.

The larger shoot tips employed in our experiments (4 x 3.5 mm) had a higher MC, lower concentration of sucrose and glycerol, and displayed larger exo- and endothermal enthalpies during cooling and warming, in comparison to smaller ones. This could explain their lower recovery after freezing in LN, which was noted in these experiments as well as in previous similar ones (2,14).

In vitrification experiments performed with garlic shoot tips, the optimal preculture durations leading to the highest recovery percentage were 3 days at 10 °C or 1 day at 23°C . As preculture duration increased, shoot tips elongated and their physiological status as well as histological structure changed, resulting in significantly ($P < 0.05$) lower recovery percentage, as already observed in previous experiments with garlic shoot tips (2). This observation could be related to the increase in enthalpy values measured in shoot tips frozen after the longest preculture durations employed.

A significant effect of sucrose concentration in the preculture medium on exo- and endothermal enthalpies was observed both in precultured (non-dehydrated) and precultured and dehydrated (PVS3 for 150 min) shoot tips. However, no significant effect of sucrose on onset temperatures was observed in precultured and dehydrated shoot tips. In vitrification studies, it has been shown notably with garlic (2) and potato (Kim *et al*., unpublished data) that sucrose concentration in the preculture medium significantly affects regeneration of cryopreserved shoot tips. Recovery after freezing was highest in shoot tips precultured with 0.5 M sucrose, although thermal properties were similar in shoot tips after preculture with 0.1 or 0.5 M sucrose, cold acclimation, preculture and loading. We can thus speculate that the positive role of sucrose may be related to an improvement in recovery of cryopreserved shoot

tips, as well as to a direct effect on thermal events taking place in shoot tips, *e.g.* by limiting/blocking ice nucleation and ice melting.

Sucrose and glycerol concentration decreased in garlic shoot tips air-dried for 1-3 h before dehydration, compared to non-air dried shoot tips, as also observed in previous experiments (14). In the vitrification protocol established, air-drying for 1-3 h before PVS3 dehydration resulted in poor recovery of cryopreserved garlic shoot tips, as noted previously (12). However, air-drying before dehydration did not significantly modify the thermal events recorded during cooling and warming of garlic shoot tips. These observations indicate that the lower recovery observed after freezing is due to desiccation damage caused by air-drying before dehydration as well as to lower permeation of cryoprotectants.

Only small crystallization/ice melting peaks were recorded in shoot tips dehydrated with PVS1, PVS2, Steponkus and Fahy solution. It is thus highly likely that the lower recovery of both dehydrated control and LN stored garlic shoot tips dehydrated with these solutions for 120 min observed during these experiments as well as in previous ones (12) is due to cytotoxicity of cryoprotectants rather than to crystallization/ice melting events. The nature of cytotoxicity can be biochemical and/or osmotic (20). Osmotic stress itself may not be responsible for the lower recovery noted in our experiments, since the MC of shoot tips dehydrated with these solutions was between 56.5-66.7 % after 120 min, which was significantly higher than that of shoot tips dehydrated with PVS3 (35.6 %) after the same duration. Therefore, the cytotoxicity of these solutions is likely due to chemical toxicity.

Large thermal events were still recorded in shoot tips dehydrated for 120 min with Towill solution, which contains a lower concentration of cryoprotectants (23), compared with the other solutions employed. The lower regeneration of cryopreserved shoot tips dehydrated with Towill solution for 120 min thus is due to both cytotoxicity and detrimental crystallization events. Melting enthalpy of dehydrated shoot tips is higher than that of freezing enthalpy, remarkably so in Towill solution, which means devitrification and recrystallization occurred within the treated samples during warming.

The duration of dehydration with PVS3 significantly influenced all thermal parameters recorded as well as recovery of cryopreserved shoot tips. The various combinations of pretreatments and dehydration procedures tested also significantly modified thermal events recorded in shoot tips. This whole set of experiments performed with garlic shoot tips confirms, as already observed with numerous materials (9,10), that recovery after freezing increases in line with a decrease in the intensity of crystallization/ice melting events in shoot tips.

Our study has shown that the various parameters studied, *i.e.* sucrose and glycerol concentration, thermal events (enthalpy, onset temperature) and MC of shoot tips are well correlated with the recovery results achieved after freezing. This systematic approach helps understanding the physical and biochemical events underlying a vitrification procedure and is thus of critical importance to optimize protocols. Vertucci (25) has identified at least five types of water with different thermal properties as seed moisture content changes during desiccation. In seven species of *Coffea* studied, a significant linear correlation between the ice melting enthalpy of water and lipids and seed moisture content has been found within the higher moisture contents (12~22 % fresh weight basis) (8). In the present study, two categories of moisture content with different thermal properties could be identified, *i.e.* above and below 35% MC. At MCs of above 35 %, both enthalpy and onset temperature significantly decreased linearly as shoot tip MC decreased. At MCs below 35%, enthalpy did not significantly change, while onset temperature drastically decreased. At optimum condition a few of samples recorded small crystallization and melting enthalpies. Cooling and warming velocity of shoot tips plunging into LN is considerably faster than those DSC running, *i.e.* 10°C per min. Thus, crystallization and melting enthalpies of actual samples cryopreserved may be smaller than those DSC running.

In conclusion, our study confirms that thermal analysis is very useful tool for optimizing cryopreservation protocols, if some factors (*e.g.* cytotoxicity) are carefully controlled.

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