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EVOLUTION OF DMSO CONCENTRATION IN GARLIC SHOOT TIPS DURING A VITRIFICATION PROCEDURE

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Abstract

In this paper, the evolution of dimethylsulfoxide (DMSO) concentration and moisture content (MC) of garlic shoot tips was studied during the course of a vitrification protocol using the PVS2 vitrification solution. DMSO concentration of shoot tips increased rapidly, reaching 34.1 mg/g fresh weight after 20 min of PVS2 treatment and remained stable afterwards, while moisture content decreased from 82 to 60 %, reaching 53 % after 60 min. A reverse process was observed during unloading. There was a highly significant negative correlation between shoot tip moisture content and DMSO concentration during the dehydration and unloading treatments. Using unloading solutions with osmolarities between 0.42 and 2.29 Osm led to very different shoot tip MCs, between 63.55 and 81.24 %, while DMSO concentration was between 14.83 and 19.97 mg/g fresh weight. After 24 h on recovery medium, DMSO concentration of shoot tips had decreased to 3.2 mg/g fresh weight.

Keywords: garlic, shoot tips, DMSO, HPLC, PVS2, vitrification.

INTRODUCTION

The vitrification solutions employed in the vitrification-based procedures developed for freezing plant materials are mixtures of cryoprotectants, which have been formulated for their ability to vitrify upon freezing, *i.e.* to form an amorphous glassy structure (7). Among the vitrification solutions developed for freezing plant tissues, PVS2 (Plant Vitrification Solution N° 2) designed by Sakai *et al.* (22), has been successfully applied for freezing shoot tips of over 200 species/cultivars (21). PVS2 is composed of (w/v) 30 % glycerol, 15 % dimethylsulfoxide (DMSO) and 15 % ethylene glycol (EG) in culture medium with 0.4 M sucrose. Its total molarity is 7.8 M, and it is thus highly toxic to plant tissues. The duration and temperature of application of PVS2 have thus to be determined very precisely (20, 25). It

is also imperative to remove PVS2 immediately after rewarming through an unloading treatment in order to avoid its toxic effect, as illustrated recently with garlic shoot tips (13).

Cryoprotectants are classified based on their permeability to biological samples. DMSO, glycerol, EG and sucrose have been classified as penetrating since they permeate the cell wall and plasmalemma (5, 27). DMSO is one of the most widely employed cryoprotectants for animal, human and plant tissues. DMSO permeates cells very rapidly, even at low temperature, and has a good glass-forming tendency (7, 28). However, because of the short duration of exposure of explants to the PVS2 solution, it is considered that its constituent cryoprotectants do not have time to penetrate the cytosol and that they have only an osmotic action (20, 22, 23, 24).

Various techniques are available to measure the penetration of cryoprotectants inside biological samples, including ^{14}C labelled radiotracer methodology (4), high pressure liquid chromatography (HPLC) (2), proton nuclear magnetic resonance (NMR) spectroscopy (8) and microscopy (12). However, the kinetics of influx and efflux of cryoprotectants in/out of the samples during a cryopreservation protocol have been little investigated.

Successful cryopreservation of garlic shoot tips using the PVS2 and/or PVS3 solutions has been reported from laboratories in several countries (3, 9, 10, 13, 15, 19). In our laboratory, garlic is used as a model material to study the various parameters involved in the establishment of cryopreservation protocols for shoot tips of bulbous crops.

In this study, we followed the evolution of DMSO concentration in garlic shoot tips during the course of a vitrification protocol using the PVS2 vitrification solution.

MATERIALS AND METHODS

Plant material

The Korean garlic cv. Eiusung was used in this study. This cultivar is classified as cold-region type garlic. Bulb scales were planted in Suwon, Korea in September 2000 in plastic greenhouses with mulching provided by black plastic film. Bulbs were harvested at the end of June 2001, 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) until sampling. Eiusung being a cold-region type variety, the bulbs showed deep dormancy. From late August 2001 onwards, shoot tips of some cloves started growing after inoculation *in vitro*. Experiments started in early September 2001, when dormancy had been broken in most of the clove shoot tips, and finished by mid-February 2002. After these dates, most of the shoot tips had sprouted and displayed low survival after cryopreservation.

Sample preparation and surface sterilization

Shoot tips were extracted from garlic cloves using borers with a diameter of 3.0 mm. The basal plates were cut with a scalpel (No. 11 blade) until they were 0.5 to 0.8 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 with commercial household detergent for at least 5 min, 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 in a laminar air flow cabinet.

Vitrification procedure

Shoot tips were precultured at 10 °C for 3 days on full strength Murashige & Skoog (MS, 15) medium with 0.1 M sucrose under a photoperiod of 16 h light/8 h dark, with a light intensity of 60 µmol/m^2/s. Shoot tips were then dehydrated for 20 min in PVS2 solution (30 % (w/v) glycerol + 15 % (w/v) DMSO + 15 % (w/v) EG in MS with 0.4 M sucrose) (22), with 30 shoot tips in 30 ml PVS2 solution, under constant shaking at 23 °C. A few minutes before freezing, shoot tips were transferred to 2 ml cryovials (Nalgene Co., 30 shoot tips per cryotube), which were filled with 1 ml fresh PVS2 solution and plunged directly in liquid nitrogen (LN). Cryopreserved samples were stored in LN for 24 h before rewarming. Rapid rewarming was achieved by plunging samples in a water-bath thermostated at 37 °C until the cryoprotectant solution became liquid. The PVS2 solution was then removed and replaced with liquid medium containing 1.28 M sorbitol, in which the explants were kept for 40 min. For regrowth, shoot tips were cultured on MS medium supplemented with 0.04 mg/l kinetin + 0.2 mg/ gibberellic acid (GA_3) + 3 % sucrose, solidified with 0.25 % gelrite, at 23 °C and under a photoperiod of 16 h light/8 h dark, with a light intensity of 60 µmol/m^2/s. Regeneration of shoot tips was evaluated by counting the number of shoot tips which had developed leaves 30 days after cryopreservation (3).

The effect of the following parameters on the moisture content (fresh weight basis) and DMSO concentration of shoot tips was investigated:

- Size of shoot tips, which varied between 1.50 and 3.75 mm in diameter and 3.0 and 3.5 mm in length (Table 1). Shoot tips were extracted from cloves with borers of the corresponding diameter.
- Duration of dehydration treatment with PVS2, from 0 to 60 min (Fig. 2).
- Concentration of the PVS2 solution, which varied from 113 % to 60 % (Fig. 3). Full strength PVS2 solution was diluted with culture medium containing 0.4 M sucrose until the desired concentration was reached.
- Duration of unloading treatment in medium with 1.28 M sorbitol, from 0 to 60 min (Fig. 4).
- Composition of the unloading solution, which included 0.66, 1.28 or 1.76 M sorbitol, or 0.3 or 1.2 M sucrose in liquid medium (Table 2). The osmolarity of the solutions was measured with an Advanced Micro-osmometer (Model 3300, Advanced Instruments Inc., USA). The instrument was calibrated with 50 and 850 mOsm/l standards before use and each test solution was assayed in duplicate or triplicate.
- Air dehydration (1 h in a laminar air flow bench) or treatment with a loading solution (medium with 0.8 M sucrose + 1 M glycerol or 0.4 M sucrose + 2 M glycerol) (Table 3).

Analysis of DMSO concentration

For sample preparation, shoot tips were rinsed quickly in distilled water and smoothly blotted dry on filter paper. A minimum of 1.1 g shoot tips (63-65 shoot tips) was placed in a glass vial and 10 ml of 10 % methanol were added per g of fresh matter. The vial was then tightly capped and left overnight at room temperature for DMSO extraction. All samples were diluted as needed in 10 % methanol and centrifuged for 10 min at 5,000 rpm. The HPLC method employed was a modification of that described by Carpenter and Dawson (2). The mobile phase contained 65 % methanol isocratic (v/v) in high purity deionised water (resistance > 18 MΩ) and was degassed by helium sparging. The flow rate was 1.0 ml/min, and the detector (Varian 9012) was set at 214 nm (UV). The column used in this study was a Supercosil LC-18 (25 cm × 4.6 mm, particle size 5 µm). The volume injected was 5 µl.

An aliquot of the shoot tips extract was sampled, diluted to 1/10 with 10 % methanol and assayed for measurement of DMSO content using the HPLC method outlined above. A

sample of PVS2 solution with a known DMSO concentration was used as standard. The standard was diluted to 1/10 with 10 % methanol to simulate the dilution resulting from the addition of methanol to shoot tips extracts. This preparation was then further diluted to 1/10 with 10 % methanol, centrifuged and assayed as described above.

To determine the DMSO concentration in the aqueous phase of shoot tips samples, the following equation was used:

Sample concentration = DMSO 1000 ppm (mg/ml) x sample area / standard area x sample dilution factor.

Statistical analysis

DMSO concentrations are presented as mg/g fresh weight ± standard deviation. Results were analyzed using the least significance difference (LSD) and Duncan's multiple range (DMRT) tests, with the SAS 8.1 software. Experiments were replicated 2-3 times.

RESULTS

Regeneration of cryopreserved shoot tips ranged between 12-57 % (data not shown). The highest regeneration percentages were obtained with shoot tips sampled from *in vitro* plantlets cold-acclimated at 5 °C for 2-3 weeks, loaded with 0.4 M sucrose + 2 M glycerol for 30 min and dehydrated with PVS2 solution for 30 min.

In the DMSO standard, the absorbance peak was observed at 214 nm (Fig. 1). Neither glycerol nor ethylene glycol (EG) were detected within the wavelength range of 200 - 400 nm (data not shown).

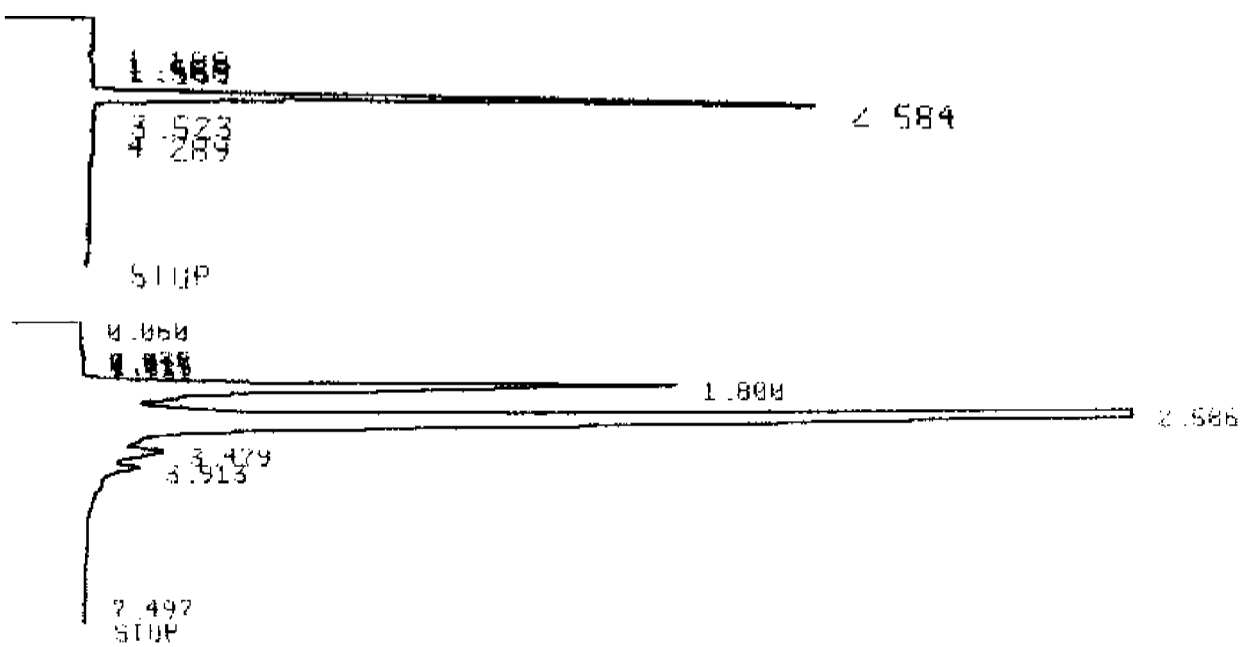

Figure 1. Diagrams of DMSO standard (1,000 mg/l) (top curve) and of DMSO in garlic shoot tips after loading with 0.8 M sucrose + 1 M glycerol and dehydration with PVS2 for 20 min (bottom curve).

As shoot tips diameter increased, their MC after PVS2 treatment increased from 51.4 to 60.3 %, while the DMSO concentration decreased from 41.3 to 38.0 mg/g FW (Table 1).

Table 1. Moisture content (% MC, fresh weight basis) and DMSO concentration (mg/g FW) of garlic shoot tips as a function of their size (diameter × length, mm). Shoot tips were dehydrated with PVS2 for 20 min. Figures followed by the same letter are not different at the 99 % level based on LSD.

	Dimensions of shoot tips (diameter x length, mm)			
	1.50 x 3.0	2.25 x 3.0	3.00 x 3.0	3.75 x 3.5
MC (%)	$51.4 \pm 0.8_a$	$55.8 \pm 0.7_b$	$58.6 \pm 0.6_c$	$60.3 \pm 0.7_d$
DMSO (mg/g)	$41.3 \pm 4.5_a$	$39.7 \pm 4.1_b$	$38.1 \pm 4.0_c$	$38.0 \pm 4.2_c$

The kinetics of DMSO penetration in shoot tip tissues displayed a bi-phasic nature, with a rapid initial influx followed by a slower increase (Fig. 2). More than half of the total DMSO quantity entered shoot tips within 5 min. During the same period, shoot tip MC decreased drastically from 80 to 70 % and then more slowly, reaching 53 % after 60 min. Shoot tip MC and DMSO concentration were highly (negatively) significantly correlated (r = -0.961).

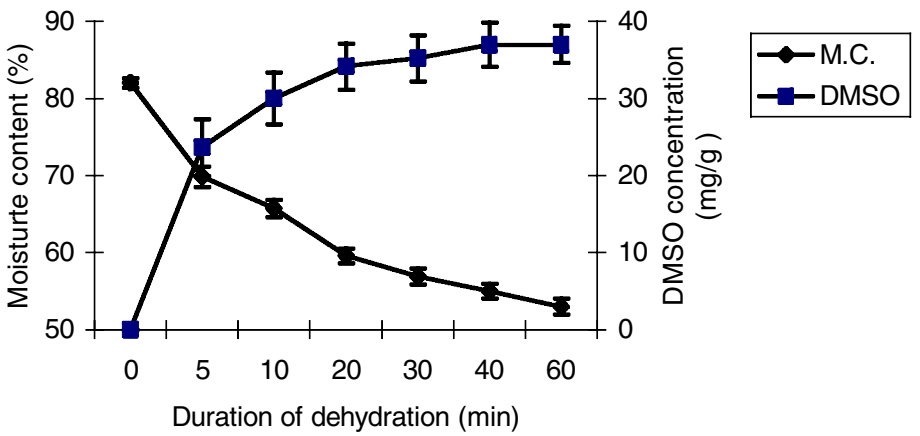

Figure 2. Evolution of moisture content (% MC, fresh weight basis) and DMSO concentration (mg/g FW) of garlic shoot tips as a function of the dehydration duration (min) with PVS2 solution.

Shoot tip MC varied between 62.5 % and 55.0 % and DMSO concentration between 31.5 mg/g and 41.3 mg/g after treatment with 60 % and 113 % PVS2, respectively (Fig. 3). There was a highly significant negative correlation (r = -0.996) between MC and DMSO concentration of shoot tips.

Shoot tip MC increased rapidly during the first 5 min of the unloading treatment, then more slowly and remained stable from 20 min onwards, indicating that equilibrium had been reached (Fig. 4). DMSO concentration decreased rapidly within 5 min, from 31.09 to 19.96 mg/g, and then more slowly, reaching 10.18 mg/g after 60 min. A highly significant negative correlation (r = -0.962) was found between MC and DMSO concentration of shoot tips.

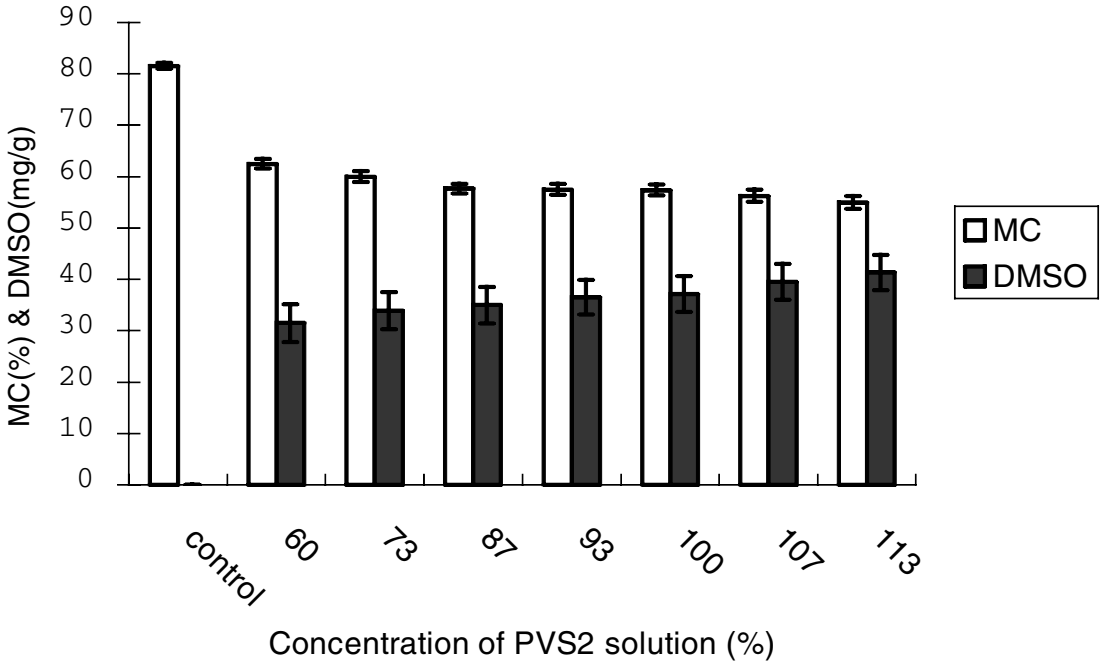

Figure 3. Moisture content (% MC, fresh weight basis) and DMSO concentration (mg/g fw) of garlic shoot tips as a function of the concentration of the PVS2 solution. Precultured shoot tips were treated with different strength of PVS2 for 20 min.
Control: non-dehydrated control.

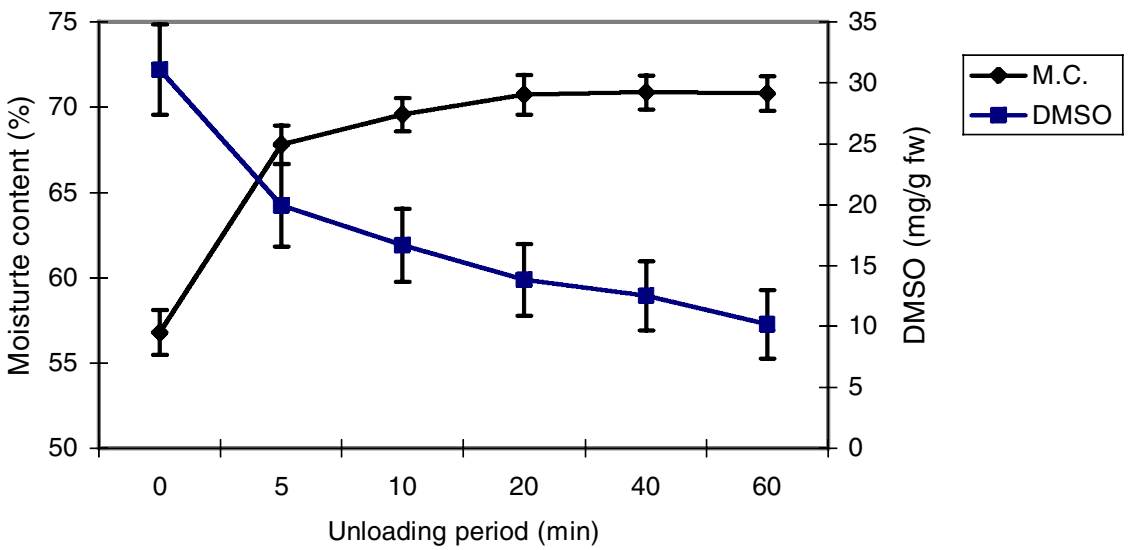

Figure 4. Evolution of moisture content (% MC, fresh weight basis) and of DMSO concentration (mg/g FW) of garlic shoot tips as a function of the duration of the unloading treatment in liquid medium with 1.29 M sorbitol.

The osmolarity of the unloading solutions employed varied between 2.29 Osm (1.2 M sucrose) and 0.42 Osm (0.3 M sucrose) (Table 2). Shoot tip MC was 53.78 % immediately after rewarming and it varied between 81.24 % and 63.55 % after unloading with 0.66 M sorbitol and 1.20 M sucrose, respectively. Despite the large differences noted in osmolarity of unloading solutions and shoot tip MC at the end of the unloading treatment, only slight differences were observed in DMSO concentration, which varied between 19.97 mg/g (1.2 M sucrose) and 14.83 mg/g (0.66 M sorbitol).

Table 2: Effect of the solution employed during unloading treatment (40 min) on moisture content (% MC, fresh weight basis) and DMSO concentration (mg/g FW) of garlic shoot tips. Figures followed by the same letter are not different at the 99 % level based on DMRT.

	Unloading solution					
	Control*	Sorbitol 0.66 M	Sorbitol 1.29 M	Sorbitol 1.76 M	Sucrose 0.30 M	Sucrose 1.20 M
Osmolarity (Osm)	-	0.71	1.46	2.24	0.42	2.29
MC (%)	$53.8 \pm 1.0_f$	$81.2 \pm 1.4_a$	$70.3 \pm 1.3_c$	$66.8 \pm 1.3_d$	$80.0 \pm 1.4_b$	$63.6 \pm 1.4_e$
DMSO (mg/g)	$38.1 \pm 3.2_a$	$14.8 \pm 2.9_e$	$17.5 \pm 3.1_d$	$18.0 \pm 3.2_c$	$17.3 \pm 3.0_d$	$20.0 \pm 3.2_b$

*Control: MC and DMSO concentration were measured immediately after rewarming, before unloading treatment.

Whatever the treatment applied to shoot tips before dehydration, their MC after PVS2 treatment was similar, comprised between 54.8 and 57.4 % (Table 3). Shoot tip MC increased to 70.9 % after unloading and retrieved its original value after 1 day culture on recovery medium. The DMSO content of shoot tips dehydrated with PVS2 was also very similar, comprised between 32.33 and 35.32 mg/g, whatever the treatment applied before dehydration, except in the case of shoot tips which had been air-dehydrated before PVS2 treatment. The DMSO content of such shoot tips was only 8.92 mg/g. After unloading, shoot tips contained 12.48 mg/g DMSO. After 1 day culture on recovery medium, the DMSO content of shoot tips had decreased sharply, reaching 3.19 mg/g.

DISCUSSION

This paper provided original information on the evolution of DMSO concentration in garlic shoot tips during the course of a vitrification protocol using the PVS2 vitrification solution. The main findings of this study were as follows: DMSO penetration in shoot tips and its efflux from shoot tips were rapid, since constant shoot tip DMSO concentrations were reached within several tens of minutes of PVS2 or unloading treatment. After the same PVS2 treatment duration, larger shoot tips had higher MC and lower DMSO concentrations than smaller ones. Air-drying of shoot tips before PVS2 treatment blocked the penetration of DMSO into shoot tips. After 40 min unloading treatment in various sucrose- or sorbitol-based solutions, the DMSO concentration of shoot tips was 1.9 to 2.5 times lower than in shoot tips transferred directly on recovery medium after rewarming.

Table 3. Moisture content (% MC, fresh weight basis) and DMSO concentration (mg/g FW ± SD) of garlic shoot tips after various treatments. Figures followed by the same letter are not different at the 99 % level based on DMRT.

Treatment†	MC‡ (%)	DMSO (mg/g)
Control	82.1	0.0_{f}
PC → DH	82.3 → 57.4	$32.33 \pm 2.38_{b}$
PCS → DH	78.1 → 56.2	$32.84 \pm 2.64_{ab}$
AD → DH	69.4 → 56.3	$8.92 \pm 1.99_{d}$
LD1 → DH	63.0 → 54.8	$35.32 \pm 2.46_{a}$
LD2 → DH	66.6 → 55.3	$34.64 \pm 2.57_{ab}$
DH → UnLD	57.4 → 70.9	$12.48 \pm 2.06_{c}$
DH → PoC	57.4 → 81.7	$3.19 \pm 0.31_{e}$

†Control: Shoot tips immediately after inoculation *in vitro*; PC, Preculture with 0.1 M sucrose at 10°C for 2 days; PCS, Preculture with 0.5 M sucrose at 10 °C for 2 days; LD1, Loading with 0.8 M sucrose + 1 M glycerol for 60 min; LD2, Loading with 0.4 M sucrose + 2 M glycerol for 60 min; AD, Air-drying under laminar air-flow cabinet for 1 h; DH, Dehydration with PVS2 for 20 min; UnLD, Unloading with 1.28 M sorbitol solution for 40 min; PoC, Post-culture on medium with 0.1 M sucrose for 1 day. ‡: the left and right figures correspond to the initial and final shoot tip moisture content, respectively, for each of the treatments tested.

The kinetics of DMSO penetration during a cryopreservation protocol has been studied notably with animal tissues. During cryoprotective treatment of porcine islets, DMSO penetration displayed a biphasic nature (2) or a non-linear time-dependent pattern (11), with an initial rapid influx followed by a more gradual increase in DMSO concentration. A similar pattern has been observed in this study. Delays in penetration of DMSO into the core region, compared to periphery, also imply the reason for concentration of DMSO into larger shoot tips were lower than into smaller ones.

These new data on the evolution of DMSO concentration in garlic shoot tips during their treatment with the PVS2 vitrification solution provide explanations for some of the results obtained recently with the cryopreservation of garlic shoot tips using a series of vitrification solutions (3, 13). The higher survival and regeneration percentages of smaller shoot tips compared to that of larger ones (3) might be related to their lower MC and higher DMSO concentration. The occurrence of an optimal explant size for freezing has been demonstrated with various materials, including notably banana (29), taro (26), cassava (6) and chrysanthemum (17). Larger explants have a more heterogeneous histological composition and comprise more differentiated and hydrated tissues in which lethal crystallization occurs during freezing, which impedes regrowth of whole samples (22). In addition, penetration of cryoprotectants into the core of the explants and efflux of water might be slower than in the external cell layers (2).

The lower amount of cryoprotectants penetrating shoot tips submitted to an air-dehydration treatment before the PVS2 treatment could explain the lower survival after cryopreservation of shoot tips submitted to such treatment, compared to that of shoot tips not submitted to air-drying (13).

Finally, the significant drop in regeneration percentage observed when cryopreserved shoot tips were transferred directly on recovery medium, without unloading treatment compared with shoot tips treated with unloading solutions (13) is clearly related to the fact that unloading decreases rapidly the concentration in shoot tips of the highly toxic PVS2 solution.

Even though DMSO penetrates both the cell wall and the plasmalemma (26), it is still not clear whether DMSO penetrated, or not, in the cytoplasm of garlic shoot tips cells during the PVS2 treatment. Sakai *et al.* (22) and Steponkus *et al.* (24) stated that neither sucrose nor glycerol could permeate in the cytoplasmic domain within such short durations of loading treatments. NMR imaging may provide useful information on the permeation of CP molecules inside the samples (1, 8, 12). Finally, it would be interesting to study the kinetics of influx and efflux of vitrification solutions constituted of other cryoprotectants.

REFERENCES

1. Bidault NP, Hammer BE & Hubel A (2000) *Cryobiology* **40**, 13-26.
2. Carpenter JF & Dawson PE (1991) *Cryobiology* **28**, 210-215.
3. Cho EG, Kim HH, Engelmann F, Baek HJ & Chae YA. *CryoLetters* **24**, in press.
4. Conniff KL (1983) MSc Thesis, Colorado State University, Fort Collins, USA.
5. Dumet D, Engelmann F, Chabrillange N, Dussert S & Duval Y (1994) *Seed Sci Res* **4**, 307-313.
6. Escobar RH, Mafla G & Roca WM (1997) *Plant Cell Rep* **16**, 474-478.
7. Fahy GM, Levy DI & Ali SE (1987) *Cryobiology* **24**, 196-213.
8. Fuller BJ & Busza AL (1994) *CryoLetters* **15**,131-134.
9. Hannan R & Garoutte D (1996) in *Proc Natl Onion Res Conf*, Madison, USA, pp101-103.
10. Hannan R & Garoutte D (1998) in *Proc Natl Onion Res Conf*, Sacramento, USA, pp292-298.
11. Hu JF & Wolfinbarger LJ (1994) *Cryobiology* 31, 461-467.
12. Ishikawa M, Ide H, Price S, Arata Y & Kitahsima T (2000) in *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Applications*, (eds) Engelmann F & Takagi H, JIRCAS, Tsukuba & IPGRI, Rome, pp22-35.
13. Kim HH, Cho EG, Baek HJ, Kim YJ, Keller ERJ & Engelmann F. *CryoLetters*, in press.
14. Langis R & Steponkus PL (1990) *Plant Physiol* **92**, 666-671.
15. Makowska Z, Keller J & Engelmann F (1999) *CryoLetters* **20**, 175-182.
16. Murashige T & Skoog F (1962) *Physiol Plant* **15**, 473-497.
17. Niino T, Seguel I & Murayama T (2000) in *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Applications*, (eds) Engelmann F & Takagi H, JIRCAS, Tsukuba & IPGRI, Rome, pp194-199.
18. Niino T, Tashiro K, Suzuki M, Ohuchi S, Magoshi J & Akihama T (1997) *Sci Hortic* **70**, 155-163.
19. Niwata E (1995) *CryoLetters* **16**, 102-107.
20. Sakai A (2000) in *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Applications*, (eds) Engelmann F & Takagi H, JIRCAS, Tsukuba & IPGRI, Rome, pp1-7.
21. Sakai A (2003) in *Proceedings of the International Workshop on Cryopreservation of Bio-Genetic Resources*, International Technical Cooperation Center, Rural Development

Administration, 3-5 June 2003, Suwon, Korea, (eds) Cho EG & Kim HH, National Institute of Agricultural Biotechnology, Suwon, pp3-18.
22. Sakai A, Kobayashi S & Oiyama I (1990) *Plant Cell Rep* **9**, 30-33.
23. Sakai A, Kobayashi S & Oiyama I (1991) *J Plant Physiol* **137**, 465-470.
24. Steponkus PL, Langis R & Fujikawa S (1992) in *Advances in Low Temperature Biology Vol 1*, (ed) Steponkus PL, JAI Press Ltd, Hampton Mill, UK, pp1-61.
25. Takagi H (2000) in *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Applications*, (eds) Engelmann F & Takagi H, JIRCAS, Tsukuba & IPGRI, Rome, pp178-193.
26. Takagi H, Thinh NT, Islam OM, Senboku T & Sakai A (1997) *Plant Cell Rep* **16**, 594-599.
27. Tao D & Li PH (1986) *J Theor Biol* **123**, 305-310.
28. Taylor R, Adams GDJ, Boardman CFB & Wallis RG (1974) *Cryobiology* **11**, 430-438.
29. Thinh NT, Takagi H & Yashima S (1999) *CryoLetters* **20**, 163-172.

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