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Cryopreservation of Asparagus (*Asparagus officinalis* L.) Cultured *in vitro*

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Abstract
Cultured cells and somatic embryos derived from the mesophyll tissues of asparagus (*Asparagus officinalis* L.) were cryopreserved by vitrification. The plant vitrification solution (PVS) contained 22% (w/v) glycerol, 15% ethylene glycol, 15% propylene glycol and 7% DMSO in Murashige-Skoog (MS) medium enriched with 0.5 M sorbitol. The highest survival rates of vitrified cells and embryos were about 65 and 50%, respectively. Surviving embryos developed into plantlets. Single node segments of asparagus were desiccated and successfully cryopreserved in liquid nitrogen. Preculture for 2 days on MS medium containing 0.7 M sucrose was found to be effective for cryoprotection. Precultured segments were then dried at 25°C until their water contents reached a level of about 20%. Dried segments were directly immersed in liquid nitrogen from room temperature, and then rewarmed in the ambient air at 22°C. The highest survival rate, as determined by shoot formation, was 63%. There was no callus formation. Complete plantlets were regenerated from shoots.

Discipline: Biotechnology
Additional key words: desiccation, sucrose, vitrification

Introduction

Cryopreservation of plant cells, embryos and meristems can be an important tool for long-term preservation of germplasm or experimental materials because it prevents the occurrence of genetic alterations during preservation. Successful cryopreservation of cultured plant cells and organs has been based on the avoidance of detrimental intracellular ice crystal formation during the cooling and rewarming processes. When cells are sufficiently dehydrated, the intracellular solutes become concentrated enough to undergo vitrification (formation of an amorphous solid) during cooling, and to sustain subsequent rewarming without being damaged by significant recrystallization, under ordinary cooling and rewarming conditions. Consequently, almost every cryopreservation protocol which has been reported so far (freeze-dehydration, dehydration with highly concentrated cryoprotectants, or dehydration in the ambient air with a low humidity) includes a dehydration process before the materials are quenched in liquid nitrogen (LN_2). In those protocols which incorporated freeze-dehydration, cells were frozen to about -40°C slowly enough to prevent the formation of intracellular ice crystals prior to immersion in LN_2[9,17]. Cryopreservation procedures which employ dehydration with concentrated cryoprotective solutions are referred to as vitrification, and have recently been applied to several different species[11]. In the air-drying method, materials are dehydrated in the ambient air with or without alginate encapsulation. Fabre and Dereuddre[4] reported on the cryopreservation of *Solanum* shoot-tips using this encapsulation-dehydration technique.

The present paper attempts to review the results of studies on the development of procedures for the successful cryopreservation of cultured cells and organs of asparagus.

Vitrification

Normally, when a liquid is quenched in LN_2 and

becomes a transparent solid, it is considered to have vitrified, as evidenced by the whitening that occurs due to the development of ice crystals during subsequent warming, or devitrification[14]. We used PVS (plant vitrification solution; 22% (w/v) glycerol, 15% propylene glycol, 15% ethylene glycol, 7% DMSO, and 0.5M sorbitol in MS medium) for cultured asparagus cells and somatic embryos[15]. The survival rate of the cultured cells after addition and removal of 85% PVS without cooling to -196°C was 49.9% of that of the untreated control (Table 1). Furthermore, the survival rate of the cells which had been vitrified and subsequently diluted was 47.4%, indicating that vitrification and subsequent rewarming of cells did not cause any additional loss beyond that associated with the addition and removal of 85% PVS. Thus, it is obvious that while vitrification itself is not harmful to cells, the addition and removal of the vitrification solution can exert deleterious effects. Toxicity and osmotic injury due to the vitrification solution are influenced by the concentration, duration and temperature of treatment, and subsequent dilution procedures. Although we were able to control these factors, the maximal survival rates (about 65% for cultured cells and about 50% for embryos) were not sufficiently high, probably due to dehydration by PVS. In our experiments, the survival rates varied considerably from 65 to 20% depending upon the growth stage of the cell culture. Thus, to improve the rate of survival, the optimal cell growth stage[12], and more suitable procedures for vitrification must be determined.

A 2-min exposure to -70°C after rapid cooling in LN_2 did not cause any decrease in survival, but a 60-min exposure at -70°C or 10-min at -50°C resulted in a dramatic decrease in the survival rates (Table 1). In addition, the survival rates decreased considerably between -60 and -40°C during slow rewarming. These results indicate that a large number of fine intracellular ice crystals (innocuous intracellular ice) which are formed during the devitrification process damage the tissues as they develop, and that a rapid increase in the rate of crystal development occurs between -60 and -40°C, as reported in very hardy cells by Sakai[9] and Sakai and Yoshida[10].

Globular somatic embryos were also cryopreserved by vitrification (Table 2). Many asparagus plants regenerated from vitrified embryos. In somatic

Table 1. Effects of temperature on the survival of vitrified cells

Treatment		Survival (%)
Experiment 1		
Treated control[a]		47.4±3.2
Vitrified cells[b]		49.9±0.9
Experiment 2		
Supercooled at	-70°C for 20 min	42.8±2.2
〃	-70°C for 2 min[c]	41.1±3.9
〃	-70°C for 60 min[c]	6.1±0.7
〃	-50°C for 10 min[c]	9.5±1.3

a): Cell suspension was treated with 85% PVS and diluted without cooling to -196°C.
b): Vitrified cells were rewarmed directly in a bath at 22°C.
c): Vitrified cells were rapidly transferred to a bath at -70 or -50°C and kept there before being rewarmed.

Table 2. Survival rates of vitrified somatic embryos

Treatment	Survival (%)
85% PVS	48.4±6.0
100% PVS	40.3±4.5

Survival rates were determined by FDA staining after warming and dilution. Somatic embryos: early globular stage.

embryos of orange[7] which were cooled to -196°C by the conventional freezing method, recovery was not due to the survival of a whole embryo, but rather to the recovery of proliferating structures from surviving cells by secondary embryogenesis. In vitrified asparagus embryos, plantlets developed from revived embryos, which is critical for germplasm preservation.

Air-drying

Axillary buds are generally the most suitable materials for *in vitro* plant propagation, and are also useful materials for cryopreservation[3,16]. Single node segments, which consist of a piece of stem bearing a lateral bud, are commonly used for the micropropagation of asparagus[2], potato[5], and other species. In preliminary experiments, preculture on MS medium containing high sucrose concentrations[8] was found to be effective for preventing desiccation injury. The survival rate after immersion in LN_2 at a water content of about 15% was 49.5% when the preculture lasted for 2 days, but 0% without

Table 3. Effects of preculture on single node segments of asparagus

	Untreated control	Preculture for 2 days
Water content (%FW)	84.7±2.9	68.4±3.9
Dry weight per segment (mg)	1.0±0.3	2.7±0.5
Survival after immersion in LN_2 without drying (%)	0	13.2±1.7
Survival after immersion in LN_2 with a water content of 15%	0	49.5±11.5

Table 4. Concentration of soluble sugars in stem segments during preculture

Sugars	Concentration (%FW)	
	Without preculture	With preculture
Glucose	0.36±0.07	1.26±0.22
Fructose	0.17±0.04	1.04±0.03
Sucrose	3.89±0.48	7.30±0.19
Maltose	0.46±0.09	0.66±0.03

Segments were precultured on solidified MS medium containing 0.7 M sucrose for 2 days in light at 25°C.

preculture. The water content of single node segments of asparagus before preculture was 84.7%. During the 2-day period of preculture on MS medium which contained 0.7 M sucrose, the water content of the segments decreased to 68.4% (Table 3). At the same time, the dry weight per segment increased from 1.0 mg to 2.7 mg. Soluble sugar contents also significantly increased during the preculture (Table 4). When the segments were directly immersed in LN_2 without desiccation, no segments of the untreated control survived, while 13% of the precultured segments developed shoots.

The effects of the water content on the survival of asparagus single node segments precultured for 2 days is shown in Fig. 1. The survival rates both before and after the immersion in LN_2 rapidly declined when the water content was less than 19%. The maximum survival rate of 63% after immersion in liquid nitrogen was obtained at a water content of 19%. Surviving segments started developing shoots in about a week, without forming any callus. Complete plantlets regenerated from shoots.

Pretreatment with abscisic acid (ABA)[13] or a high concentration of sucrose[8] has been reported to be

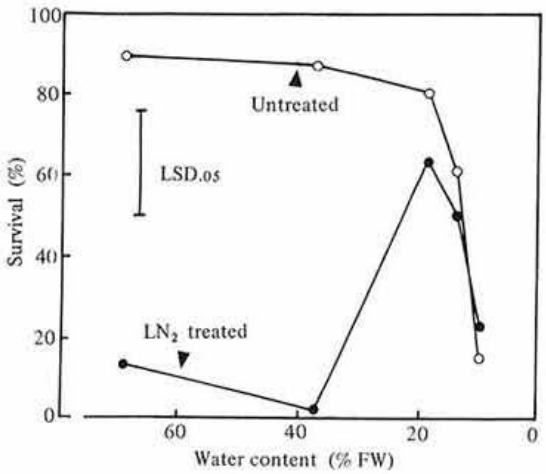

Fig. 1. Effects of water content of asparagus nodal sections on survival
○ : Before immersion LN_2,
● : After immersion LN_2.

effective for inducing desiccation tolerance in somatic embryos and callus. We selected a 2 days period of preculture with sucrose, which was much shorter than the ABA treatment, that usually requires about 2 weeks. Sucrose pretreatment had two effects: (1) It induced desiccation tolerance due to the accumulation of sucrose[6]. Sucrose absorption from the medium was confirmed by HPLC (Table 4). (2) It slowly reduced the water content by about 8% per day. This may have produced an effect similar to that of slow drying, as reported by Senaratna et al.[13].

In our study, the survival rates before the exposure to LN_2 decreased significantly at water contents below 19%, suggesting that asparagus stem segments are not fully tolerant to desiccation even after pretreatment with sucrose. After exposure to LN_2, the survival rates of the segments were maximal at a water content of 19%. The survival rates of segments before and after exposure to LN_2 at water contents below 19% were similar. These findings suggest that freezable water, which may cause freezing injury during the cooling and rewarming processes did not remain in the tissues of asparagus segments. Sakai[9] reported that when winter mulberry tree twigs were directly immersed in LN_2, slow rewarming in the ambient air destroyed all the cells due to intracellular freezing, while rapid rewarming in water (500°C per sec) had no effect. A water content of 19% is

just below the threshold moisture level of 20 to 30% which has been determined by differential thermal analysis (DTA) in other species[1].

Conclusion

Cryopreservation of cultured cells, somatic embryos and axillary buds by vitrification or air-drying seems a promising method for preserving asparagus germplasm. For the practical application of the methods described here, further studies should be carried out on the genotypic and phenotypic uniformity of the regenerants from cryopreserved materials, and on the effect of long-term storage in LN_2.

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