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APPLICATIONS OF THERMAL ANALYSIS IN CRYOPRESERVATION OF PLANT CELLS AND ORGANS

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If plant cells and organs are to survive exposure to liquid nitrogen, intracellular crystallization must be avoided. This implies preliminary dehydration of the cells before quenching in liquid nitrogen. Three main procedures have been successively proposed to ensure cryopreservation of cells and organs. In conventional procedures, dehydration of the cells results from extracellular freezing of the cryoprotective medium during the first step of cooling to –40°C. In dehydration procedures, the loss of water is generally achieved after encapsulation of shoot-tips and somatic embryos in alginate beads (synthetic seeds) by evaporation at room temperature. In the vitrification procedure, dehydration is obtained by placing plant cells and organs in extremely concentrated solutions of permeating and/or non-permeating cryoprotectants. Thermal analysis shows that the two last procedures led to glass transitions of both organs and cryoprotective media, during cooling as well as during rewarming. It appears to be a useful approach for improvement of cryopreservation by perfecting the composition of cryoprotective mixtures.

Keywords: biotechnology, organs, plant cells, cryopreservation, vitrification

Introduction

Germplasm preservation for plant breeding is generally ensured by storage of dehydrated seeds (i.e. orthodox seeds). However, for plants producing seeds which cannot resist dehydration (i.e. recalcitrant seeds), and for vegetatively propagated plants (potato, date and oil palms, banana, cassava, sugar cane), specific techniques are required. Field genebanks and *in vitro* methods can be used for short- and medium-term storage [1]. For long-term storage (several years), only ultra-low temperatures (–196°C) may ensure long-term preservation of material under conditions of good

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genetic and physiological stability. Cryopreservation can be applied to plant cell cultures and to plant organs (shoot-tips and somatic embryos).

In biotechnology, cryopreservation is proposed for storage of metabolite-producing cell lines, organogenic cell cultures, transgenic organisms and artificial seeds (shoot-tips or somatic embryos trapped in Ca–alginate).

Until recently, conventional procedures used in cryopreservation were mainly empirical. The aim was to establish an equilibrium during prefreezing, between extracellular ice and solutions remaining in the cells. This may ensure survival of cells after quenching in liquid nitrogen and subsequent thawing.

Whereas cryopreservation of cell suspension cultures can be used routinely without major problems, difficulties still remain when freezing organized and macroscopic structures (shoot-tips and somatic embryos). These difficulties will be resolved by better understanding of mechanisms involved in liquid-solid transitions during freezing and thawing.

Conventional procedures

To ensure survival of plant cells during quenching in liquid nitrogen, and subsequent rewarming to room temperature, it is necessary to prevent the formation of intracellular ice crystals by dehydration of the cells before immersion in liquid nitrogen. This procedure leads to the vitrification of intracellular solutions and implies the removal of freezable water (i.e. free water from the cells without deleterious alterations of cell structures.

The conventional procedure (Fig. 1) initially involves equilibrium of plant cells and organs in medium containing cryoprotectants. Cryoprotective media generally contain binary solutions of sucrose or polyols (0.1 M to 1 M) and DMSO (5 to 15%). Two ternary mixtures have been also proposed for cryopreservation: 0.5 M DMSO + 0.5 M glycerol + 1 M proline or sucrose [2] and 10% polyethylene glycol (PM 6 000), 8% glucose and 10% DMSO [3].

Loss of water from the cell is induced during prefreezing (freeze-induced cell dehydration) by the formation of ice in the cryoprotective medium and the resultant difference in water potentials between intra- and extracellular media. Generally, survival depends on the cooling rate during the first cooling step (generally from 0 to –40°C). The optimum cooling rate generally ranges between 0.5 and 2 deg/min, and depends on the ability of water to leave the cell and to crystallize in the surrounding cryoprotective medium.

Pre-freezing is arrested at the desired temperature and the material is quenched in liquid nitrogen. During the second step of cooling, from the prefreezing temperature (–40°C in most cases) to –196°C, residual unfrozen water is believed to turn into glassy water. During thawing, recrystallization can be avoided by rapid thawing in a water bath at +40°C.

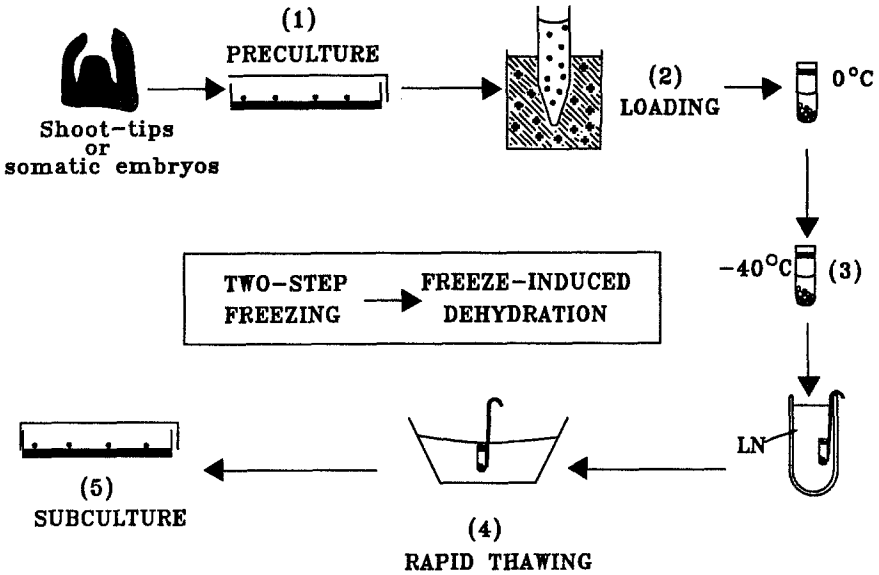

Fig. 1 Conventional procedure for cryopreservation. This comprises three main steps: pretreatment, freeze-thaw cycle and post-treatment. Cell suspensions, shoot-tips or somatic embryos are first precultured (1) under sterile conditions for 1 to 2 days on medium supplemented with sucrose or DMSO. After loading with DMSO, glycerol or other penetrating agents (2), they are transferred to cryotubes and progressively cooled to –40°C (3). After storage in liquid nitrogen, they are rapidly thawed in a water bath at +40°C (4) and subcultured on standard culture medium for reactivation (5)

Cryopreservation of cell suspensions appears to be easier than cryopreservation of organs. Although more than 70 species have been cryopreserved in the form of cell suspensions, only a few species have been cryopreserved in liquid nitrogen in the form of meristems [4] or embryos [5]. Cryopreservation of shoot-tips and embryos appears more critical than cryopreservation of cell suspensions. To ensure direct organogenesis from shoot-tips cryopreserved in liquid nitrogen, survival of the apical dome as a whole, or a major part of it, is necessary. Otherwise, callus differentiation may occur and give rise to subsequent adventitious organogenesis.

Cryopreservation of somatic embryos also appears more critical than cryopreservation of shoot-tips and implies survival of the two apical meristems and of hypocotyl tissues. Until the last two years, regrowth generally occurs by secondary somatic embryogenesis. Direct regrowth (i.e. without intermediary callus formation) is observed only with young embryos [6]. Older embryos (late-heart or torpedo stages) survive only partially, due to their size, and form calli.

Additional research is needed to improve direct resumption of organogenesis of shoot-tips and to obtain direct regrowth of somatic embryos. Two new procedures have

been successively proposed which may replace freeze-induced cell dehydration by dehydration at room temperature (Fig. 2).

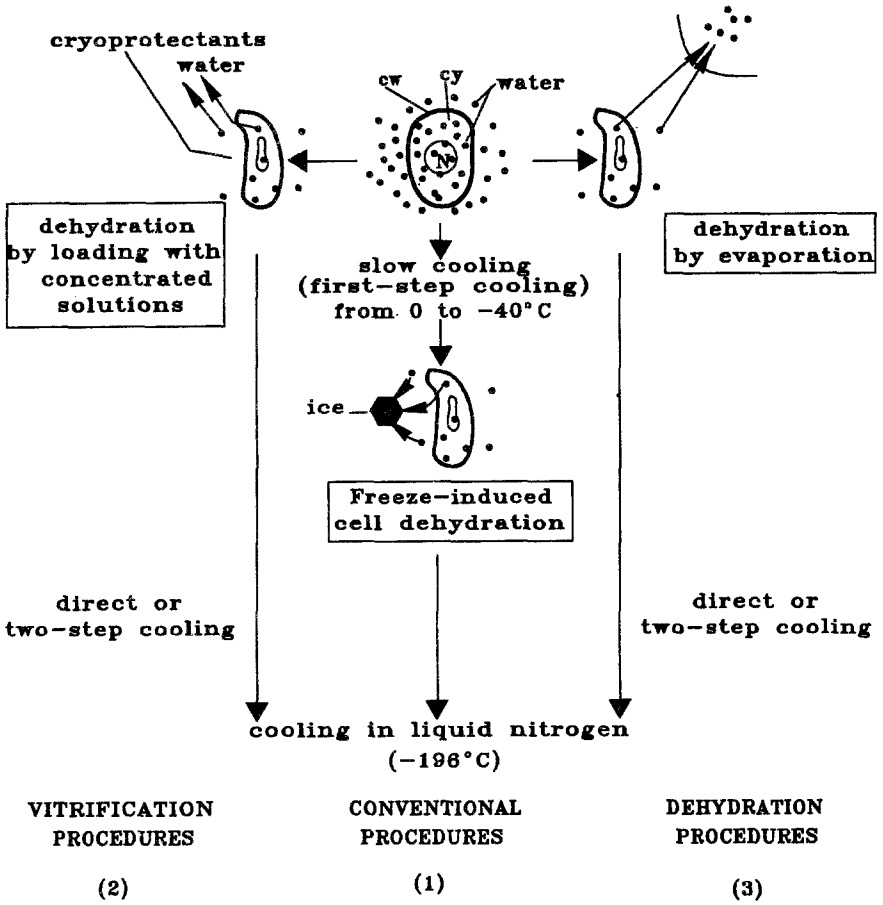

Fig. 2 Procedures proposed for cryopreservation of plant cells and organs. In the conventional procedure (1), dehydration is ensured during first-step freezing from 0 to −40°C by transfer of intracellular water to the cryoprotective medium, where it crystallizes. In the vitrification procedure (2), dehydrations is induced by progressive addition of highly concentrated solutions of cryoprotective agents. In the dehydration procedures (3), cellular water is removed by evaporation at room temperature. cy, cytoplasm; cw, cell wall; N, nucleus

Dehydration procedures

The first procedure may be referred as encapsulation-dehydration [7]. It involves coating of plant organs in alginate beads (artificial seeds). Shoot-tips or somatic

embryos were suspended in calcium-free culture medium containing 3% Na-alginate salt. The mixture was dispensed from a pipette with a sterile tip into culture medium supplemented with 100 mM calcium chloride (Fig. 3).

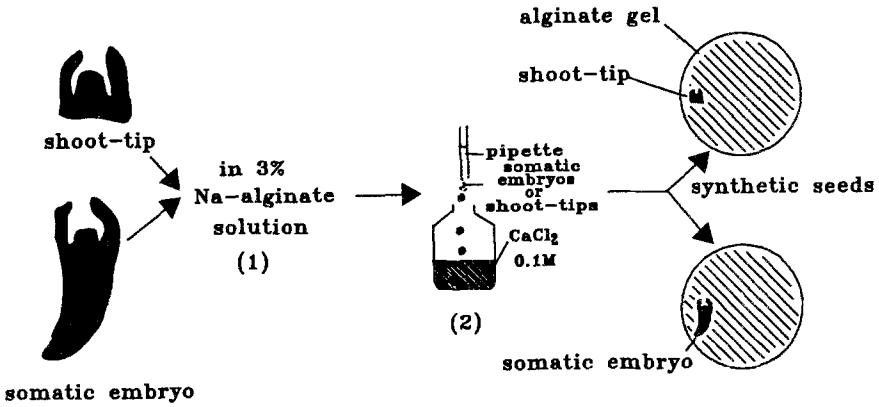

Fig. 3 Preparation of synthetic seeds. Shoot-tips or somatic embryos are suspended in 3% Na-alginate and dripped into medium containing 100 mM calcium chloride. Beads (4 mm in diameter) containing plant organs are formed by rapid gel production in the presence of Ca^{2+} ions

Beads of about 4 mm in diameter and containing 1–3 organs, were precultured in culture medium enriched with sucrose. Trapped shoot-tips or somatic embryos were dried in the sterile air of a laminar air flow cabinet at normal room temperature and humidity. After 4 to 6 hours of dehydration, the beads were transferred into cryobiological ampoules for freezing.

Two types of cooling have been used: rapid cooling by direct immersion of ampoules in liquid nitrogen, and two-step cooling including programmed prefreezing. Deep-cooled synthetic seeds were rewarmed in air at room temperature (Fig. 4).

This procedure has been successively applied to shoot-tips of several species (Fig. 5): pear [7], *Solanum* [8], carnation (unpublished results) and grape [9]. This procedure also allowed survival of somatic embryos [10]. With carnation (Tannoury, unpublished results), survival was independent of cooling and rewarming rates. As after vitrification, this property may be correlated with vitrification of both encapsuled material and the alginate matrix [11]. Dehydration may also be performed without encapsulation [12].

Vitrification procedures

In the dehydration procedures, loss of water depends on the temperature of sterile air flow and on its relative humidity, and the final water content depends on the duration

of dehydration. To avoid the effects of partly uncontrolled parameters, a second procedure has been proposed. It consists of placing cells and organs in extremely concentrated solutions of permeating and/or non-permeating cryoprotectants. This so-called vitrification technique was first applied to protoplasts [13] and cell suspensions or calli [14–17], and has also been used for plant organs [18–20].

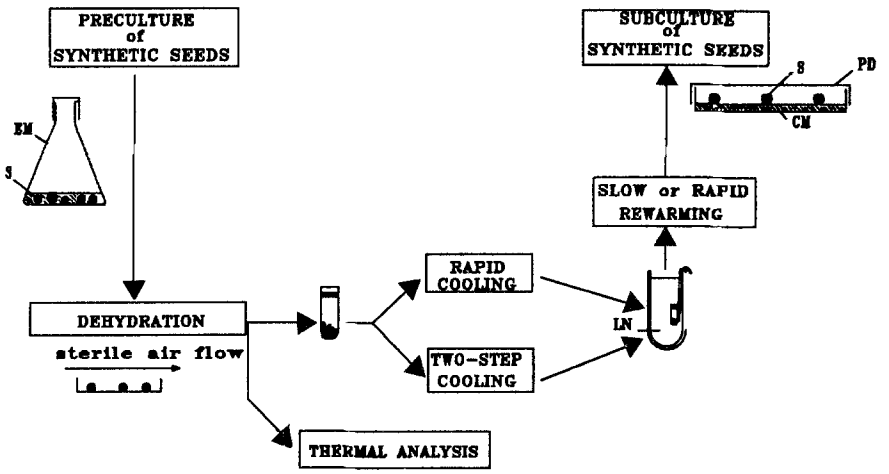

Fig. 4 Encapsulation-dehydration procedure. Shoot-tips or somatic embryos are first encapsulated in alginate beads. After overnight preculture with sucrose in erlenmeyer flasks (EM), synthetic seeds (S) are dehydrated for 4–6 hours in sterile air flow in Petri dishes, placed in cryotubes and cooled in liquid nitrogen (LN). After rewarming they are subcultured in Petri dishes (PD) on culture medium (CM)

For carnation [20], shoot-tips were first encapsulated in alginate beads and precultured for one night in medium enriched with sucrose and transferred progressively in vitrification solution containing 6 g sucrose/ 4 g water/ 6 g ethyleneglycol. With this technique, survival rates were independent of the cooling rate from 0.5°C to 200°C/min. This property could be related to the glass transition of shoot-tips during cooling and rewarming. Vitrification after loading with medium 6-4-6 may replace air-dehydration.

Thermal analysis

To study the mechanisms involved in the two non-conventional procedures, thermal analysis was performed using differential scanning microcalorimetry. The aims of this study were to compare the behaviour of cryoprotective solutions and biological material

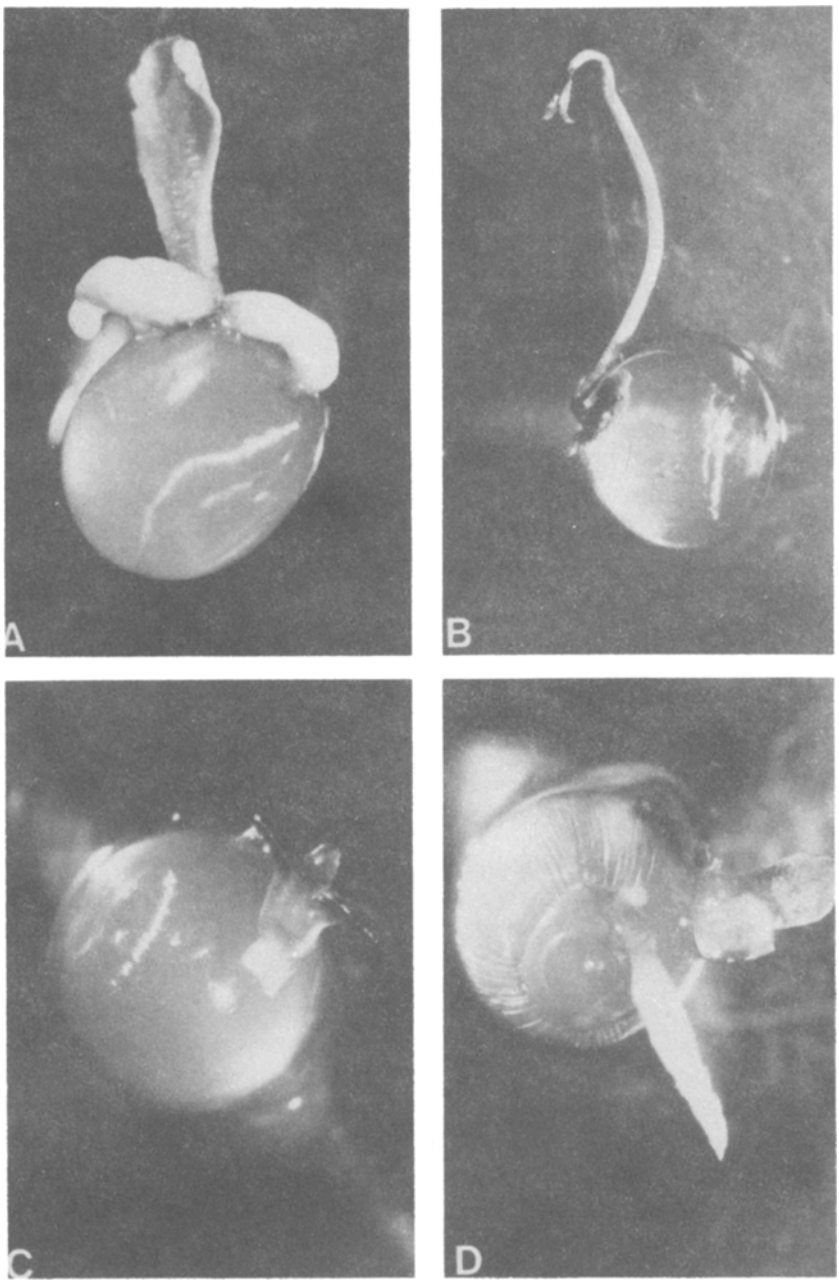

Fig. 5 Plantlet recovery from shoot-tips of pear (A), *Solanum* (B), carnation (C) and somatic embryo of carrot (D) after encapsulation, dehydration in air, cooling in liquid nitrogen and slow rewarming

submitted to a freeze-thaw cycle and to determine the conditions which can allow glass transitions to occur during cooling and rewarming.

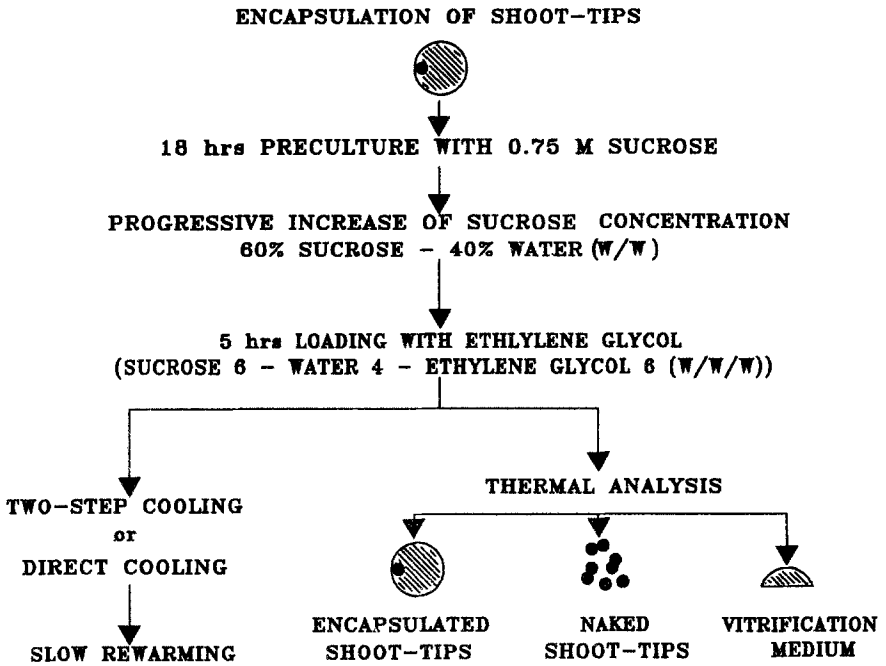

Fig. 6 Vitrification procedure for carnation shoot-tips. After overnight preculture, encapsulated shoot-tips are transferred into medium progressively enriched with sucrose until the desired concentration of sucrose (6 g sucrose/ 4 g water) is reached. They are then loaded for 5 hours at 0°C with ethylene glycol at a final concentration of 6 g sucrose/ 4 g water/ 6 g ethylene glycol. Encapsulated shoot-tips may be cooled in liquid nitrogen directly or after prefreezing at –150°C

When cells are frozen in liquid nitrogen, samples are twice submitted to temperatures which facilitate changes of state (crystallization and melting, vitrification and devitrification). Generally, it is during thawing that intracellular solutions can devitrify and crystallize if warming is not fast enough or if dehydration is insufficient. However, because of the volume of solutions and plant organs used in cryobiology, application of the vitrification procedure appeared difficult until relatively recently. To obtain glass transitions independently of cooling and warming rates, the use of concentrated solutions was suggested [21]. Hence, the problem of the resistance of cells and organs to liquid nitrogen changed to one of their tolerance to dehydration or loading with extremely concentrated solutions. Simplification of the cryopreservation procedure implies that survival is independent of cooling and warming rates; it requires true vitrification of both cryoprotective solutions and plant cells.

Any crystallization is preceded by the formation of ice nuclei. In the absence of agents of nucleation, the number of nuclei which appear spontaneously in the liquid phase increases with decreasing temperature, whereas the rate of crystal growth increases with rising temperature [21]. So, while the amorphous state can be obtained easily during fast cooling in liquid nitrogen, the stability of intra- and extracellular amorphous solutions is more difficult to maintain during rewarming.

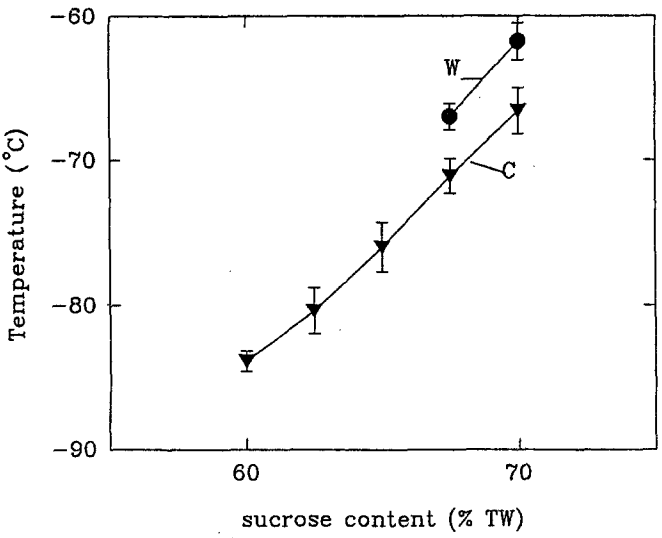

Fig. 7 Change in middle glass transition temperatures during cooling (C curve) and rewarming (W curve) as a function of the concentration of sucrose (% of total weight). During rewarming, glass transition without recrystallization is only obtained with 70% sucrose

The stability of the amorphous state (i.e. the absence of crystallization) is dependent on the temperature during the isothermal stage and on rewarming rate during thawing [22]. In cryobiology, the stability of the amorphous state can be defined by the critical warming rate above which no recrystallization occurs before the melting point [22]. It can also be defined for a given solute which allows glass transition to occur during cooling as well as during warming, whatever the cooling and warming rates. This definition may be extended to more sophisticated cryoprotective mixtures.

With sucrose solutions used as cryoprotective mixtures, the solid-phase diagram takes the form of a simple eutectic system [21, 23]. Below the temperature of the eutectic point (−13°C), the solution can crystallize. However, the increase in viscosity of the solution reduces ice nuclei formation and growth of crystals and promotes glass formation [21]. On the other hand, with supersaturated solutions, temperature decrease does not lead systematically to the crystallization of the solute: many sugars and polyols, especially sucrose, do not crystallize easily and the solution become supersaturated.

Vitrification can be obtained with a low risk of recrystallization if the concentration of the solution is higher than that for which the homogeneous temperature curve may join the glass temperature curve.

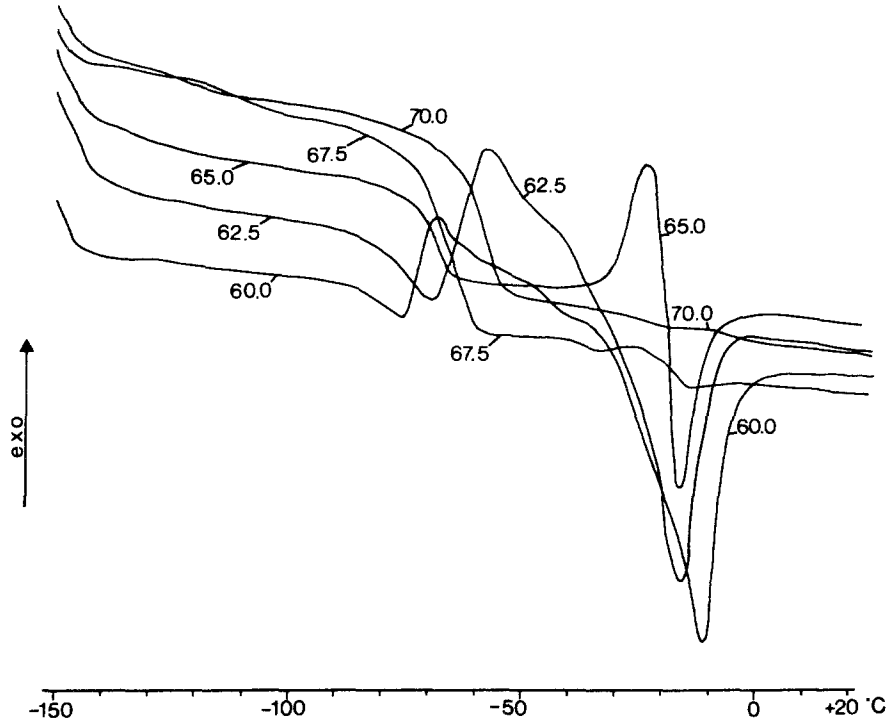

Fig. 8 Warming curves of sucrose solutions containing (% total weight): 69%, 62.5%, 65%, 67.5% and 70% sucrose. Three events are apparent: glass transition, an exothermic peak, corresponding to the crystallization of the liquid solution formed during devitrification, and an endothermic peak of melting of ice previously formed. Glass transition without recrystallization was only obtained with 70% sucrose

The water content of the vitrification solution is often considered as the unfreezable water content of the medium. It is close to 0.56 g of water per g of sucrose [21]. However, this value is not enough to ensure devitrification without ice formation during thawing. During cooling (Fig. 7), vitrification can easily be obtained with sucrose concentrations ranging from 60 to 70% (% total weight). The vitrification temperature (middle glass temperature transition) varies according to the concentration of the solute from –83°C (60% sucrose) to –66°C (70% sucrose). On rewarming, thermograms display generally three events (Fig. 8): a glass transition with an inflexion point corresponding to the transition of amorphous solid water to hypercooled liquid water, an exothermic peak corresponding to the crystallization of the hypercooled water (devitrification peak), and the endothermic melting peak of ice. The size of the two

peaks decreases as the concentration of sucrose increases. Glass transition (at – 63°C) without further recrystallization is only obtained with highest concentration (70% sucrose), corresponding to solution containing about 0.43 g of water per g of sucrose (i.e. an osmolality of about 7).

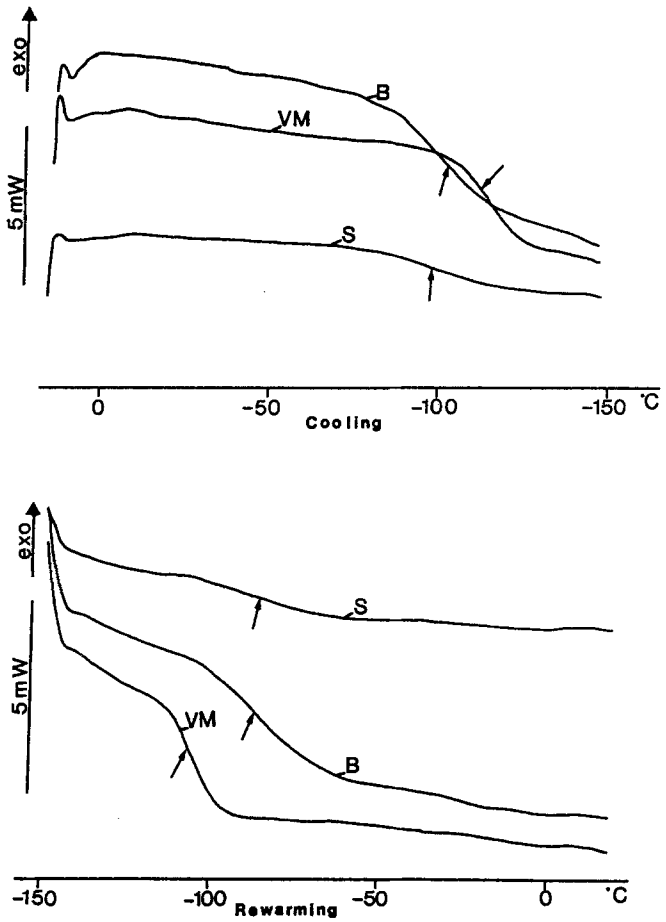

Fig. 9 Curves of cooling from +20°C to –150°C and rewarming from –150°C to +20°C of vitrification medium (VM), beads treated with loading medium (B), and carnation shoot-tips extracted from the beads (S). Cooling and rewarming rates were 10 deg/min. Vertical bars represent 5 mW, arrows indicate the middle temperature of glass transitions [20]

Glass transitions were also obtained with the vitrification solution used for cryopreservation of carnation shoot-tips (Fig. 9). During cooling as well as during rewarming, thermograms of the vitrification solution (VM) displayed a glass transition

during cooling as well as during rewarming, without any peak of crystallization or melting. The temperature of the middle point of glass transitions was equal to – 111°C during cooling and – 106.8°C during rewarming. Glass transitions were also obtained with encapsulating material and shoot-tips extracted from the beads after loading for 5 hours in vitrification solution. Glass transition temperatures of encapsulating material were equal to – 97.6 and to – 88.1°C during cooling and rewarming respectively. These two temperatures were similar to those obtained with shoot-tips extracted from beads after loading with the vitrification medium: – 94.0°C and – 88.1°C.

Conclusions

Two procedures may be proposed to replace the conventional procedure for cryopreservation of plant cells and organs. Dehydration by evaporation with or without encapsulation has been successfully applied to shoot-tips or somatic embryos of several species [8–12]. Good results have also been obtained after vitrification in liquid medium [13–20].

When the water content of sucrose solutions dropped to 30% or less, cooling and rewarming thermograms displayed glass transitions without any peak of crystallization or melting. Similar cryobehaviour has been observed with alginate beads precultured with sucrose solutions and dehydrated for several hours [11]. Vitrification of encapsulating material has also been obtained after incubation with vitrification solution containing sucrose and ethylene glycol. It seems that water contents of plant organs (shoot-tips or somatic embryos) that optimise survival in liquid nitrogen are in the range 20 and 30% [10, 12, 24].

The resistance of plant cells to dehydration is generally induced by overnight preculture with sucrose. The exact role of sucrose is still unknown. Like other cryoprotective agents (DMSO, polyols), it seems that sucrose modifies the structure of water by the formation of hydrogen bonds [21]. It is also well known that cryoprotective compounds such as polyols and sugars promote glass transition during cooling and subsequent rewarming. This property has been described with glycerol [22], 1, 2-propanediol [25], ethylene glycol [26], linear polyols [27] and galactose [28]. In terms of water-protein relations, two types of water can be distinguished: free water molecules and water molecules bound to peripherical sites on the surface or inside proteins. Water molecules bound to proteins which exhibit a reduced freezing point are referred to as unfreezable water (0.3 to 0.5 g H_2O g^{-1} polymer).

If cells can tolerate the osmotic stress needed for glass transition to occur, they should be protected: phase transitions, and consequently the coexistence of the two phases, crystalline and liquid, can be avoided.

The encapsulation-dehydration process displays several advantages: easier handling of organs, simplification of cryoprotective media, elimination of costly programmed freezers, independence of survival from cooling rates, and increased size of explants

surviving liquid nitrogen storage. This technique may be useful in practicle for cryopreservation of shoot-tips or somatic embryos sensitive to freezing of cryoprotective medium.

Like Fahy [29], we consider vitrification procedures to have benefits over conventional procedures for tissue and organ cryopreservation. Vitrification can be obtained after loading with vitrification medium or after dehydration by evaporation. However, research is still needed to decrease the toxicity of vitrification solutions and to extend preliminary results described here to other species. Progress may be possible through improved understanding of the behaviour of solutions in relation to the cytological and biochemical responses of cells to dehydration. To study the behaviour of cryoprotective solutions and biological material during a freeze-thaw cycle, differential scanning calorimetry (DSC) appears satisfactory for the moment. Independently of the measurement of enthalpy, microcalorimeters allow controlled cooling and rewarming of biological specimens. DSC allows rapid study of potential cryoprotective solutions for biological material, especially plant organs. It also allows the detection of phase transitions in plant cells and organs (mainly glass transitions) and facilitates the understanding of the mechanisms involved in the resistance of cells to ultralow temperatures.

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Zusammenfassung — Sollen Pflanzenzellen oder Organe eine Behandlung mit flüssigem Stickstoff überleben, muß eine intrazelluläre Kristallisation verhindert werden. Dies umfaßt eine vorherige Dehydratation der Zellen, bevor sie in flüssigen Stickstoff eingetaucht werden. Für eine erfolgreiche Kryokonservierung von Zellen und Organen werden drei Hauptmethoden vorgeschlagen. Bei herkömmlichen Verfahren ergibt sich die Dehydratation der Zellen aus einem extrazellulären Gefrieren des kryoprotektiven Mediums beim ersten Schritt der Abkühlung bis –40°C. Bei Dehydratationsverfahren wird der Wasserverlust ganz allgemein nach Einkapseln von Keimspitzen und somatischen Embryos in Alginatperlen (synthetisches Samenkorn) durch Verdampfen bei Raumtemperatur erreicht. Beim Anschmelzungsverfahren wird die Dehydratation erreicht, indem man die Pflanzenzellen und Organe in stark konzentrierte Lösungen von durchdringenden und/oder nichtdurchdringenden Kryoprotektanten gibt. Die Thermoanalyse zeigt, daß die beiden letzteren Verfahren zu einer Glasumwandlung sowohl der Organe als auch des kryoprotektiven Mediums führt, sowohl beim Abkühlen als auch beim Wiedererwärmen. Die Verbesserung der Zusammensetzung von Kryoprotektantgemischen scheint ein nützliche Weg zur Weiterentwicklung der Kryokonservierung zu sein.