REVIEW

Cryopreservation of Plant Cell Cultures[1]

Ursula Seitz[2]

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Abstract: The main topics of this brief overview of the cryopreservation of plant cell cultures are (a) the principles behind cryopreservation procedures as a basis from which the appropriate method for various different cultures can be developed, and (b) the question of how far the characteristics of cell strains are preserved during the freeze-thaw cycle. Of all the species successfully cryopreserved to date, only seven have been investigated with regard to their biochemical capacities. In all these cases the cultures have been shown to retain their growth patterns and biochemical traits. Furthermore, results are available which indicate that this is also valid for long-term storage. Some details are presented on the accumulation of natural products and the biotransformation of cardenolides in frozen-thawed cell cultures. On the other hand, before it is possible to recommend universally applicable cryopreservation protocols, we must better understand the cellular events which determine the freeze-tolerance of plant cell cultures.

Introduction

Without doubt, the plant kingdom is endowed with a tremendous and versatile biosynthetic capacity and, from the beginning of history, it has been a major source of medicines, seasonings, and scents. In recent years, the number of reports have increased which demonstrate that cell cultures are capable of synthesising specific products at levels equivalent to or higher than the plant from which they were derived. This topic has recently been reviewed by Staba (1). Consequently, attempts have been made to develop industrial applications for these cultures. There are several reasons for looking to plant cell cultures for an alternative to natural product synthesis. These are, among others: (a) a shortening of the growth period, (b) the elimination of the need for herbicides and pesticides, (c) constant product quality, and (d) adaptability of production volume to demand.

The routine techniques of culture maintenance are expensive and time-consuming. Typically, a cell suspension culture would need to be transferred every 7–10 days, and a callus culture every 14–30 days. Furthermore, the maintenance for cultures in the fast-growing state bears the risk of possible loss through contamination or equipment failure.

Besides these technical aspects, it should be kept in mind that plant cells growing in the dedifferentiated state are genetically unstable. Instability has been documented to occur at the karyotypic, morphological, and biochemical levels. It involves polyploid and aneuploid changes, structural changes in chromosome morphology, and mitotic abberrations. Bayliss (2) pointed out that chromosomal instability in tissue culture is the rule rather than the exception. The majority of these changes occur during culture. For example, callus lines which were derived from isolated single cells have been shown to contain diploid, tetraploid, and aneuploid cells. These could only have arisen during culture subsequent to the single cell cloning process.

With regard to the production of specific compounds, cell cultures are known which displayed great stability and yet others which are extremely unstable. *Morinda citrifolia* was shown to retain its synthetic capacity for anthraquinone accumulation during 7 years of cultivation (3). Several other species can be assigned to the group of stable cultures, for example *Coleus blumei* (rosmarinic acid) (4), *Coptis japonica* (isoquinoline alkaloids) (5), *Berberis species* (protoberberine type alkaloids) (6), and some strains of *Catharanthus roseus* (indole alkaloids) (7). On the other hand, *Peganum harmale* (harman alkaloids) (8), *Solanum laciniatum* (solasodin) (9), and *Nicotiana tabacum* (nicotine) (10) show relatively high instabilities. It is perhaps too great a simplification to establish just two categories, stable and unstable. For example, the biotransformation capacity of *Digitalis lanata* cell cultures is subjected to fluctuations but its decline during long-term cultivation is relatively slow (unpublished data). In some cases it is possible to enhance stability by repeated selection (11, 12). At any rate, especially in long-term culture, the risk of losing metabolic activity is always present.

Storage Methods

From the facts stated so far, the necessity for evolving efficient storage methods for plant tissue cultures is obvious. The approaches to storage can be divided into those where the culture is maintained in the growing state and those involving the suspension of all growth and metabolism. The latter is characterised by storage at the temperature of liquid nitrogen, or in other words, cryopreservation. For callus and shoot-tip cultures there is more than one way of limiting growth. The most widely used is a reduction in the culture temperature. Additionally or alternatively, retardant chemicals may be added to the culture medium. These may act osmotically, as in the case of sucrose or mannitol, or they may act on a hormonal level, as abscisic acid does. Storage periods for cultures under these conditions are in the range of 1 year, which may be satisfactory for short-term preservation. But the disadvantages of this method are that it is not applicable to cell cultures, that the hazards of continued growth are not eliminated, and that if growth is reduced beyond a certain, as yet undefined point, the culture will die.

The necessity of storage on the one hand, and the limitations of various approaches to storage on the other hand are described in detail in order to make it clear why cryopreservation

[1] Dedicated to Prof. Dr. E. Reinhard on the occasion of his 60th birthday.

[2] Pharmazeutisches Institut der Universität, Auf der Morgenstelle 8, D-7400 Tübingen, Federal Republic of Germany.

is the only adequate storage method for plant cell cultures. This review comprises two main topics: (a) the cryopreservation procedure in principle as a basis from which the appropriate protocol for various different cultures can be developed, and (b) the question of how far the characteristics of cell strains, especially biosynthetic capacities, are preserved during cryostorage.

Cryopreservation

Cryopreservation has been successfully performed with callus, shoot-tips, cell cultures, protoplasts, pollen, embryos, and anthers. In other words, this method can be adapted to all types of plant tissue culture systems. The various methods and recent progress have been reviewed in detail by Withers (13). In this paper I shall concentrate on the cryopreservation of cell cultures.

The experimental steps of the procedure can be summarised as follows: pregrowth, cryoprotectant treatment, freezing, storage, thawing, and recovery growth.

The pregrowth history of the cells is very important in several respects. It is documented that cells from the early growth phase, which have a relatively high cytoplasm to vacuole ratio, are the most tolerant to subsequent freezing. In the majority of cases, it was shown to be beneficial for the cells to cultivate them in a modified medium where they become adapted to high osmotic pressures. Medium additives may be sugar alcohols, sugars, or amino acids. The changes which occur on the cellular level during this period may be manifold: the size of the cells and the vacuoles, the flexibility and thickness of the cell walls as well as metabolic activities may all favorably affect freeze tolerance (14, 15).

Without exception, cell cultures require chemical cryoprotection. Mixtures are reported to be more suitable cryoprotectants than single compounds. A mixture containing DMSO, glycerol, and sucrose (see Table I) seems to be most widely applicable (16). Several solutes can form the third component – sucrose and proline are effective, but other sugars, sugar alcohols, and other amino acids may also be used.

For freezing, the specimens are transferred to suitable containers. For cells suspensions screw-top ampoules work best. A slow freezing rate of 1 to 2° C per min is effective for these cultures because it allows the protective dehydration, which is a prerequisite for the cells to survive the procedure. After the samples have reached a holding temperature of − 30 to − 40° C, the ampoules are plunged into liquid nitrogen, where they are then stored.

In most cases rapid thawing has been reported to be beneficial for the cells. A subsequent washing step to remove the cryoprotectant additives seems to be unnecessary and can even be deleterious. Therefore, the cell suspensions are normally spread on a semi-solid agar medium. There are a few reports where the first cell divisions can be detected a few days after thawing, but as a rule, the cells need a recovery phase of two or more weeks before they can be transferred to normal culture conditions.

It should be emphasised that the details of all of the steps of this procedure have to be optimised for each different culture strain. For example, it has been documented that a mannitol treatment over several days reduced the viability of certain cell cultures (18). In those cases a short preculture period (8–16 h) with the addition of 1M sorbitol may give satisfactory results (19). For procedures departing from the method described in Table I see Chen et al. (20), Weber et al. (21), and Butenko et al. (22).

Viability of Frozen-Thawed Cells

Viability tests (23) are a valuable means of estimating roughly whether or not a freezing experiment was successful. Table II summarises the post-thaw viabilities of several cell cultures. *Daucus carota*, a highly tolerant culture, showed viability values of 70 % or less. On the other hand, since actively growing cultures under standard conditions contain less than 100 % viable cells, this value is in the range of optimistic expectations. The experiments with *Digitalis lanata* show that great differences can occur among the strains of one species. *Panax ginseng* is an example for the influence of pregrowth treatment. Preculture of the cells in 6 % mannitol is not enough to enhance freezing tolerance sufficiently. The preculture treatment published by Butenko et al. (22) includes an increase in the sucrose concentration up to 20 % together with a decrease of temperature from + 25° to + 4° C over a period of 18 days. This kind of "cold hardening" raised post-thaw viabilities to 40 %.

Table I. Cryopreservation protocol (cell suspensions)

Pregrowth	Transfer of exponentially growing cells to medium supplemented with 3 to 6 % mannitol. Pregrowth period: 3 to 4 days.
Cryoprotection	0.5 M DMSO plus 0.5 M glycerol plus 1.0 M sucrose prepared in culture medium. Treatment: 1 h, 0–4° C.
Freezing Conditions	Cell suspension (1 ml samples) in freezing ampoules. Slow freezing at 1° C min^{-1} to − 35° C, 30 min at − 35° C, immersion in liquid nitrogen.
Storage	In liquid nitrogen.
Thawing	Rapid thawing in a water bath (+ 40° C).
Post-Thaw Treatments	No washing. Transfer of cells and suspending medium to culture over semi-solid medium.

Table II. Viability of cell cultures immediately after thawing. The tests were performed according to Widholm (23). FDA = fluorescein diacetate

Species	Protocol	Viability test	% viable cells
Daucus carota	see Tab. I	FDA	60–70
Digitalis lanata strain 287	see Tab. I	FDA	50
Digitalis lanata strain 10/56-S50-Se	see Tab. I	FDA	70
Panax ginseng A	see Tab. I	Phenosafranin	2–10
Panax ginseng B	see (22)	Phenosafranin	40

Growth of Cryopreserved Cell Cultures

The post-thaw viability of cells as determined by viability tests yields valuable information on freezing experiments. But it has

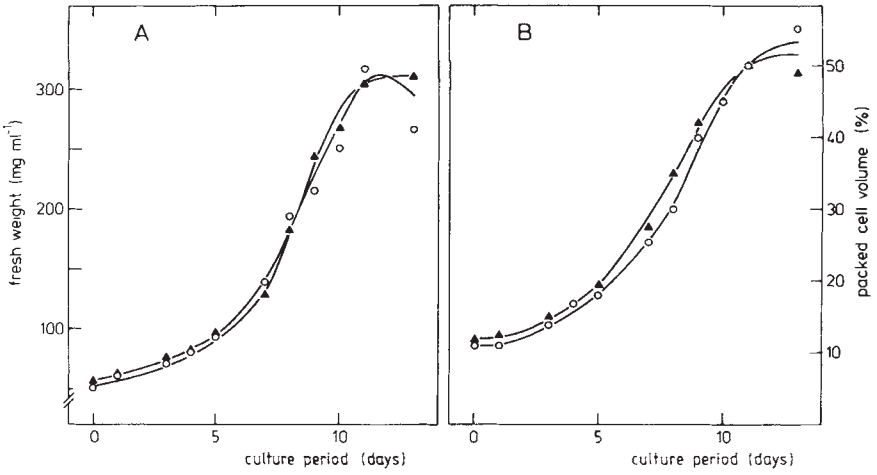

Fig. 1. Growth pattern of *Panax ginseng* cell cultures.
A: Fresh weight. Cells from 5 ml cell suspension were collected on filters, washed twice, drained under vacuum, and weighed.
B: The packed cell volume (pvc) was determined after centrifugation (10 min, 2000 rpm) of 1 ml of cell suspension in a graduated tube.
○ frozen-thawed cells, ▲ control.

been shown that frozen-thawed cells may be extremely vulnerable and that in spite of very careful handling during the early recovery phase it is often impossible to sustain viability at the initial level (18, 24). Therefore, a shift towards a quantitative evaluation of growth is suggested. It appeared important to test the growth behaviour of a culture which displayed a relatively low percentage of viable cells after thawing, for example *Panax ginseng*. Fig. 1 shows that the growth patterns expressed as packed cell volume (pvc) and fresh weight of frozen-thawed cells and of the control culture were identical. The same result was obtained with *Daucus carota* and *Digitalis lanata* cell cultures.

Cryostorage and Preservation of Biosynthetic Capacities

Cells of an Afghan variety of *Daucus carota* accumulate anthocyanin in large amounts. This culture was treated according to Table I, thawed after a storage period of 5–7 days, and cultivated for a short period on semi-solid medium and then as usual in liquid medium. The post-thaw viability is given in Table II. The anthocyanin content of a culture which had been frozen and a control which had not were then compared. The result is depicted in Fig. 2. The maximum values and the accumulation kinetics were both identical. This is in complete agreement with the results published by Dougall and Whitten (25), who investigated anthocyanin production in different strains of *Daucus carota* after freezing.

Digitalis lanata cells were frozen and thawed as described in Table I. After 3–5 passages the reestablished culture was used for biotransformation experiments. β-Methyldigitoxin was used as the substrate for this reaction. The main part of the product (β-methyldigoxin) could be obtained from the culture medium. The *Digitalis* strain from which the frozen cells had been taken was used as a control. The results are given in Fig. 3. The time curves of β-methyldigoxin accumulation from cultures treated in two separate freezing experiments were identical. A comparison with the control curve revealed that no reduction in biotransformation capacity occurred during the freeze-thaw procedure.

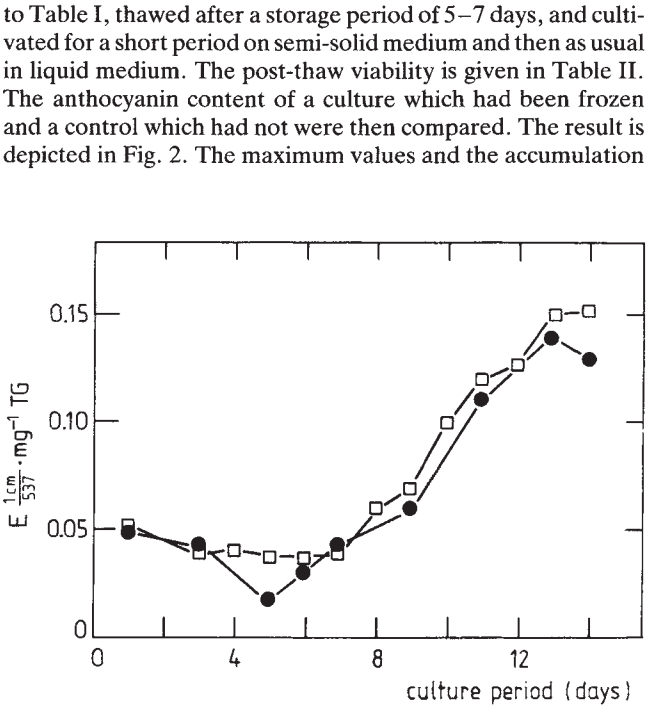

Fig. 2. Time course of anthocyanin accumulation in *Daucus carota* cell cultures. Freeze-dried material was extracted with methanol-HCl and the absorbance at 537 nm was determined.
● frozen-thawed cells, □ control.

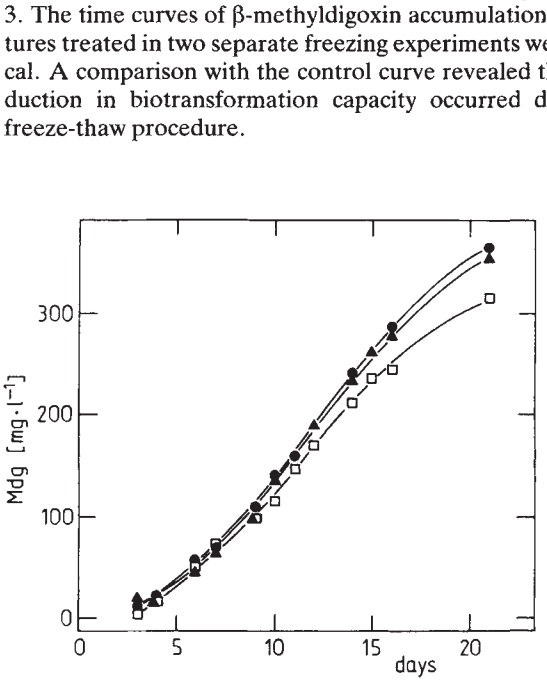

Fig. 3. Biotransformation of β-methyldigitoxin (Mdg) in a shake culture of *Digitalis lanata* (strain 287). Glucose was fed from day 8 to maintain a glucose level of between 2 and 5 $g \cdot l^{-1}$. A aliquot of culture medium was diluted with methanol and analysed by HPLC (28).
●▲ frozen-thawed cells, □ control.

Two additional species were investigated in our laboratory: *Panax ginseng* and *Coleus blumei*. The ability to biosynthesise ginsenosides and rosmarinic acid, respectively, was preserved during cryostorage [unpublished data and (19)].

A prerequisite for using cryopreservation as a long-term storage method is, of course, that the samples remain unchanged over long periods of time. Experiments were performed with *Digitalis lanata* independently from Luckner's group in Halle and in our laboratory. Our results revealed that the final yields from the biotransformation reaction are not reduced after storage periods of 2 months, 6 months, 1 year, and 2 years (data not shown). Diettrich et al. (26) have demonstrated that the capacity of *D. lanata* cells to glucosylate added digitoxin was preserved during a 3-year storage period. Moreover, the cells showed the same growth rate, DNA content, and embryogenic capacity as did the original cell population.

Table III presents a summary of reports on the cryopreservation of cell cultures which produce natural compounds or biotransform added substrates. All of the species investigated to date have been shown to retain their biochemical capacity during cryostorage.

Table III. Preservation of biochemical capacities during cryostorage

Species	Compound	Reference
Catharanthus roseus	indole alkaloids	(20)
Coleus blumei	rosmarinic acid	(19)
Daucus carota	anthocyanin	(18, 25)
Digitalis lanata	cardenolid biotransformation	(27, 28)
Dioscorea deltoidea	steroids	(22)
Lavandula vera (callus culture)	biotin	(29)
Panax ginseng	ginsenosides	(unpublished data)

Conclusions and Perspectives

An examination of the literature shows that cryopreservation has been reported on for more than 50 species; and the list is growing steadily. About 30 species can be cryostored in the form of cell cultures. For recent review of these results, see Withers (30). Despite this accumulation of data, it is difficult to recommend universally applicable storage protocols. The necessity of collecting as much information as possible on the behaviour of the cells during all of the phases of cryopreservation is self-evident, so that we come closer to the time when we can develop general methods which can be applied to a great number of cultures without the time-consuming, empirical determination of optimum conditions.

Preculture treatment is able to initiate important changes which increase the freezing tolerance of cells. A closer look at the events which occur on the cellular level may yield information which enables us to treat cultures known to possess a very low freezing tolerance.

If cryopreservation ever becomes a routine method, it could be used in the storage of plant tissue cultures with important biochemical and morphological capacities and the long-term storage of tissue culture collcections, and could facilitate the international exchange of such material.

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