Increased Survival and Differentiation of Frozen Herbaceous Plant Organ Cultures through Cold Treatment

Received for publication September 28, 1976 and in revised form January 10, 1977

MICHAEL SEIBERT AND PHYLLIS J. WETHERBEE
GTE Laboratories, Incorporated, Waltham, Massachusetts 02154

ABSTRACT

Cold treatment of donor carnation plants (Dianthus caryophyllus L.) at 4°C for 3 days or more resulted in a doubling in the percentage of excised, frozen shoot apices which survived freezing and a 6- to 7-fold increase in the percentage which formed leaf primordia or shoots. The optimal freezing parameters for both survival and differentiation were as follows: size of the shoot apex—two to three sets of leaf primordia; dimethylsulfoxide concentration in the freezing solution—5%; time in dimethylsulfoxide prior to freezing—>30 minutes; average cooling rate—2.5°C/minute; initial warming rate—about 1450°C/minute. In general, the cells in the meristematic region of the shoot apex remained viable after freezing while those in the leaf primordia did not. Viability of the meristematic cells appears to be maintained by preventing the growth of intracellular ice crystals formed during rapid cooling by rapidly passing the tissue through the temperature zone in which lethal crystal growth occurs (mechanism of Luyet). Applications of this technique are discussed.

It has become apparent that the condition of plant cells prior to freezing greatly influences their survival. For example, it is known that cold-hardened plants (1, 3) and plant tissue (18, 24) survive at lower temperatures than do unhardened plants of the same species. Furthermore, prefrozen cells (2, 20), callus (21), and woody tissue (16, 18) have much higher survival rates after fast cooling to −196°C and thawing than do untreated cells.

For these reasons, it was decided to examine the influence of donor plant cold treatment on the survival and differentiation of excised carnation shoot apices after freezing to −196°C.

MATERIALS AND METHODS

Dianthus caryophyllus L. cultivars 'Scania' and 'Linda' were obtained as rooted cuttings from commercial sources. The cuttings were planted in a commercial potting mix (Pro Mix B) containing vermiculite, perlite, and peat at high density (150 cuttings in a 2,000-cm² flat). Cold treatment was accomplished by placing flats of cuttings in an environmental chamber (Controlled Environments, Ltd., Winnipeg, Canada) under the following conditions: temperature, 4°C; photoperiod, 8 hr/day; light irradiance, cool white fluorescent—0.5 mw/cm² between 400 and 700 nm (1500 lux) plus incandescent—0.036 mw/cm² between 400 and 700 nm (80 lux).

Carnation shoot apices including the meristem and two sets of leaf primordia (unless otherwise stated) were excised from surface-sterilized shoot tips which had been peeled back to four or five sets of leaves. In order to be classified as a usable shoot apex, the ends of the second sets of leaf primordia had to cover the meristem dome and touch. Such apices were 1 to 2.5 mm long depending on the size of the older pair of leaf primordia.

Sample apices were floated in 5-ml specimen vials on 0.25 ml of a rinsing solution (22) containing Murashige and Skoog salts (12), 30 g/l sucrose, 0.4 mg/l thiamine-HCl, 100 mg/l myoinositol, 0.1 mg/l indole-3-acetic acid, and 0.5 mg/l kinetin with the pH adjusted to 5.7. At the desired time, an additional 0.25 ml of rinsing solution at twice the final desired DMSO1 concentration was added to the vial to make up the freezing solution. In Figure 5 for DMSO concentrations greater than 10% (v/v), the shoot apices were added to the freezing solution at the indicated concentration.

Samples were cooled by pouring liquid N₂ directly into the freezing vial while dipping the vial directly into an open Dewar flask filled with liquid N₂. This gave an average cooling rate of 400 to 440 C/min and a maximum cooling rate of 850 to 1100 C/min between −10 and −70°C (Fig. 1A). Microorganism contamination was not a problem. Other cooling rates (between −10 and −70°C) reported in Figure 6 were obtained by suspending experimental freezing vials over liquid N₂ and manually adjusting the distance of the vials from the N₂. The desired rate was maintained by comparing the output slope from a copper-

---

1 Abbreviation: DMSO: dimethylsulfoxide.
RESULTS

Cold Treatment. In order to determine whether or not cold treatment of donor plants affected the rate of survival of excised, frozen shoot apices, a number of plants were exposed to 4 °C in an incubator for about 7 weeks. The results from this preliminary experiment were quite encouraging since 2 weeks after subjecting 40 shoot apices to a freeze/thaw cycle, all 40 apices showed signs of survival and 24 (60%) appeared to be differentiating. This constituted a large improvement over the 30% survival rate we reported (22) for frozen shoot apices obtained from untreated plants.

Figure 3 shows the effects of shorter periods of cold treatment. Three days exposure to 4 °C was sufficient to elicit more than a doubling in the percentage of frozen shoot apices which survived freezing and a 6- to 7-fold increase in the percentage which formed leaf primordia or shoots. It should be mentioned that the rooted cuttings used in this experiment were shipped to us during the end of April. Consequently, they may have been exposed to low outside temperatures at times during the 2-day shipping period.

Size of the Apex. Since the size of the explant might influence survival and differentiation, shoot apices with from one to four sets of leaf primordia were excised from cold-treated plants. The ends of the largest leaves were removed in the case of apices containing four sets of leaf primordia so that the apices would fit into the freezing vial. Table 1 shows the effect on survival 1 week after thawing and on differentiation 1 month after thawing.

![Figure 1](attachment:image1.png)  
**FIG. 1.** Cooling curves obtained using a copper-constantan thermo-couple and a pen recorder. A: curve obtained when liquid N₂ is poured directly into the freezing vial; B: curve obtained when the cooling rate was adjusted to 10 °C/min.

![Figure 2](attachment:image2.png)  
**FIG. 2.** Warming curve obtained by placing a vial into 37 °C water.

constantan thermocouple located at the liquid surface in a dummy freezing vial with a precalculated slope on a pen recorder chart. Figure 1B shows the recorder trace for a −10 °C/min cooling rate. After the temperature reached −80 °C, the vials were plunged directly into liquid N₂. All samples were kept in liquid N₂ for at least 30 min but not more than 1 hr before warming.

Shoot apices were thawed by plunging the vials into 37 °C water. Such conditions gave an average initial warming rate of around 1,450 °C/min. (This is roughly equivalent to the 120 °C/min warming rate between −50 °C and −10 °C described by Nag and Street in ref. 15.) Figure 2 shows a pen recording of a typical warming curve. The slower initial warming rates reported in Figure 7 were obtained by warming the vials in air or blowing air past the vials. All warming curves were similar in shape to that in Figure 2.

After warming to room temperature, the thawed apices were washed twice in rinsing solution and then planted in Belco tubes (25 × 125 mm) on a growing medium consisting of the rinsing solution solidified with 1% bacto-agar (DIFCO). The tubes were placed in growing cabinets at 26 ± 2 °C with 16 hr/day exposure to light from Gro-Lux fluorescent lamps (0.43 mw/cm² from 400–700 nm or 600 lux). Shoot apices which formed callus or Chl or which showed signs of growth or differentiation were termed surviving apices. Differentiating apices were defined as those which formed new leaf primordia or shoots.

![Figure 3](attachment:image3.png)  
**FIG. 3.** Effect of cold treatment on 'Scania' carnation shoot apex survival and differentiation after freezing. Donor plants were exposed to 4 °C for the indicated number of days. After cold treatment, shoot apices (meristem and two sets of leaf primordia) were excised from the cuttings, treated with 5% DMSO for 85 min, frozen to −196 °C by pouring liquid N₂ directly into the freezing vial, thawed by placing the vial in 37 °C, washed twice in rinsing solution, and planted in growing medium. ( ): percentage of surviving apices 6 days after thawing; ( ): percentage of differentiating apices 20 days after thawing. Error bars signify 95% confidence limits.

<p>| Table 1. Effect of Shoot Apex Size on Survival and Differentiation After Thawing |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Sets of Leaf Primordia</th>
<th>Survival 1 Week</th>
<th>Differentiation 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>67 (36)</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>91 (36)</td>
<td>47</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>92 (36)</td>
<td>58</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>72 (29)</td>
<td>68</td>
</tr>
</tbody>
</table>

1 *p < 0.05 for this treatment compared with treatment 2 using the arcsin transformation test for comparing 2 binomial distributions (5).
FREEZING CARNATION ORGAN CULTURES

Fig. 4. Effect of exposure time in 5% DMSO on the survival and differentiation of cold treated 'Linda' carnation shoot apices after thawing. See Figure 3 for the experimental conditions. (●): survival 2 weeks after thawing; (●): differentiation 5 weeks after thawing. Error bars signify 95% confidence limits.

Fig. 5. Effect of DMSO concentration in the freezing solution (85 min exposure time) on the survival and differentiation of cold treated 'Linda' carnation shoot apices after thawing. See Figure 3 for the experimental conditions. (●): survival 1 week after thawing; (●): differentiation 1 month after thawing. Error bars signify 95% confidence limits.

Maximal survival rates occurred in apices containing two or three sets of leaf primordia and maximal differentiation rates in apices containing two sets of leaf primordia or more. Although the rate of differentiation for treatment 4 was statistically indistinguishable from those of treatments 2 and 3, much more callus formation was noted.

Cryoprotective Agent. The presence of a cryoprotectant, DMSO in our case, in addition to cold treatment of the donor plant was an integral requirement in order to insure high rates of survival and differentiation after subjecting carnation shoot apices to a freeze/thaw cycle. Figure 4 shows the survival and differentiation rates of frozen, cold-treated carnation shoot apices as a function of exposure time in 5% DMSO. Figure 5 plots the same parameters as a function of DMSO concentration in the freezing solution (85 min exposure time). The results show that treatment with 5% for 80 to 100 min appears optimal for both survival and differentiation.

Cooling and Warming Rates. Figures 6 and 7 show the effects of cooling and warming rates on both survival and differentiation of cold-treated, excised carnation shoot apices after freezing. Maximal survival and differentiation occur at cooling rates above 50 C/min and at the fastest warming rate tested.

DISCUSSION

The morphological stage and physiological condition of the plant material prior to freezing greatly influence its ability to survive. For example, Nag and Street (14) have shown that highly vacuolated wild carrot cells are killed under freezing conditions where small cells and aggregates of small highly meristematic cells survive. Sugawara and Sakai (25) demon-
stratified that a higher percentage of cells from 5- to 6-day-old sycamore suspension cultures survived freezing to −30°C in the presence of cryoprotective additives than did cells at any other stage of growth. At this point of development (late lag or early cell division), the cells were known to be small and dense. Our results support these observations since cell division and growth after thawing are primarily noted in the meristematic region of the shoot apex. As can be seen in Figure 8, the cells in the leaf primordia do not survive.

As was demonstrated in Figure 3, cold treatment of donor carnation plants at 4°C for as little as 3 days greatly improves the survival and differentiation rates of excised shoot apices after rapid cooling to −196°C and rewarmed to room temperature. This phenomenon is probably related in some manner to the cold-hardening process rather than the prefreezing technique (2, 16, 18, 20, 21) mentioned in the introductory section. Prefreezing increases survival by allowing for partial dehydration of the tissue at subfreezing temperature prior to rapid cooling to −196°C (20).

Table II reviews the freezing and thawing parameters which were found optimal for survival and differentiation of carnation shoot apices. With the exception of the cooling rates, the values determined for the organ cultures were consistent with those observed previously for cell cultures (4, 13-15). The rapid cooling rates which we report in Figure 6 suggest that viability is maintained by a different mechanism than that employed by investigators who freeze cell cultures at slow cooling rates (4, 13-15). The fact that faster warming rates in addition to fast cooling rates are required (Fig. 7) to observe maximal survival of carnation shoot apices suggests the mechanism of Luyet (7). Specifically, viability is maintained by preventing the growth of intracellular ice crystals formed during rapid cooling by rapidly passing the tissue through the temperature zone in which lethal crystal growth occurs. This mechanism appears to be dependent on DMSO (Figs. 4 and 5), which may decrease the growth rate of intracellular ice crystals (20). Survival of cell cultures frozen at slow freezing rates may involve some beneficial effects of dehydration which minimize the amount of intracellular water which freezes intracellularly (19) and the protective nature of DMSO.

Application of the method presented in this work as a means of preserving plant lines for extended periods of time appears promising. However, additional studies are required in order to confirm that shoot apices can remain viable in the frozen state for long periods of time and that plants obtained from frozen apices do not exhibit genetic abnormalities. Preliminary results indicate that the viability of carnation shoot apices, cooled to −196°C, does not decrease after 6 months storage at that temperature.

Acknowledgments—P. L. Stepnowski and P. B. Holliday contributed helpful discussions of this work and D. S. Raker provided technical assistance.

LITERATURE CITED


15. Nag KK, HE Street 1975 Freeze preservation of cultured plant cells II. The freezing and thawing phases. Physiol Plant 34: 261-265


