Long-term storage of mulberry winter buds by cryopreservation

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Mulberry is an important woody feed crop used for rearing silkworm in Japan, China and several Asian countries. Approximately one thousand mulberry species or cultivars are maintained at several field repositories in Japan. But conserving mulberry trees resources requires much labor and large space, so development of a new conserving method being able to replace conventional one is very important. Cryopreservation may be a useful method for long-term storage of germplasm using a minimum of space and maintenance. Prefrozen winter buds from fieldgrown mulberry trees were stored in liquid nitrogen (LN, −196°C) and regenerated plants through meristem culture (YAKUWA and OKA, 1988). We also reported that partial dehydration of winter buds before prefreezing and subsequent prefreezing to −30°C in 5°C steps at daily intervals improved the recovery rates of shoot tips of winter buds cooled to −196°C (NIINO et al., 1991).

To ascertain the long-term viability and genetic stability in LN (−196°C) or deep-freezer at −135°C, the present study was performed using mulberry winter buds.

Materials and Methods

Branches were collected from about 5–13 year-old mulberry plants (Morus bombycis Koids.) in January when the buds were still in a state of “quiescence”. The following cultivars were used for experiments: Kenmochi (mainly), Araguwa, likuwa 1, Iwate 1, Nichirenso, Nittagawa, Hayamuragawa, Haruirogawa, Yamanoobeso, Shinso 2, Hokkaiyaso, Jinbei 2 and Sumonwase.

The axillary buds with about 10 mm of vascular tissue were removed from the branch. Buds were put into a polyethylene bag and then placed in a stainless steel freezing canister (37 mm in diameter, 135 mm length) and kept at 0°C for one day before freezing. Buds were cooled in 10°C steps at daily intervals from 0°C to −30°C by successively lowering the temperature to −30°C. They were kept for 1 day prior to immersion in LN (−196°C) or before being transferred to a deep freezer at −135°C.

After storage at cryogenic temperatures for various lengths, buds were rapidly thawed in a water bath at 37°C.

Winter buds were sterilized with 70% ethanol for 1 min followed by sodium hypochloride solution (effective chlorine concentration: 0.5%) for 30 min. After rinsing in sterilized water, buds containing the meristem and five to eight leaf primordia were aseptically excised. The buds were then cultured on MS medium (MURASHIGE and SKOOG, 1962) containing 2% fructose, 0.8% agar (Wako, Ltd, Osaka, Japan) and supplemented with 1 mg/l 6-benzyl
aminopurine. The medium was adjusted to pH 6.0 before autoclaving at 120°C for 15 min. Buds were grown on 10 ml in glass vials (Wheaton 200, 24 ml). The cultures were maintained at 25°C under a 16 hr photoperiod (52 μmol m⁻² s⁻¹).

Rate of shoot formation was defined as the percentage of buds developing normal shoots 40 days after plating.

Squash preparations were made from shoot tips of in vitro plantlets, which were regenerated from winter buds immersed into LN (−196°C) or −135°C for 3.5 years. Shoot tips were fixed in ethyl alcohol-acetic acid (3:1) for 24 hr at 5°C. Fixed shoot tips were stained by the aceto-orcein method.

Results and Discussion

It is very important that cryopreserved winter buds are capable of maintaining viability during storage and of producing plants identical to the non-treated phenotype. The rates of shoot formation of winter buds stored at various temperatures for various lengths of duration are shown in Table 1. The rates of shoot formation of the buds stored in LN (−196°C) for 1 month, 6 months, 1 year and 3.5 years were 75%, 72.5%, 70.0% and 75.0%, respectively. Little or no decrease of shoot formation was observed among storage lengths in the buds stored at −135°C as those stored in LN (−196°C). However, in the winter buds stored at −40°C or −70°C, no or little recovery was observed.

The shoot formation of cryopreserved winter buds at −135°C for 3.5 years was investigated using 12 other mulberry cultivars (Table 2). High levels of shoot formation were obtained in these cultivars tested. The average rate of shoot formation was about 72%.

No morphological abnormalities were observed in the plants developed from cryopreserved winter buds stored for 3.5 years at −135°C or −196°C. The chromosome observations showed that all the 15 regenerated plants examined were diploid with 28 chromosomes regardless of the storage temperatures (Fig. 1).

SHIRATA et al. (1992) reported that no change of morphological abnormalities of characters such as leaf length, leaf width, length of branch, number of branches, lobation and others were observed in the plants regenerated from cryopreserved winter buds compared to unfrozen control.

Table 1. Rate of shoot formation of winter buds stored at various temperatures for various lengths of duration.

<table>
<thead>
<tr>
<th>Duration</th>
<th>Shoot formation rate (%±S.E.)</th>
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<tbody>
<tr>
<td></td>
<td>−40</td>
</tr>
<tr>
<td>1 month</td>
<td>0±0</td>
</tr>
<tr>
<td>6 months</td>
<td>0±0</td>
</tr>
<tr>
<td>1 year</td>
<td>—</td>
</tr>
<tr>
<td>3.5 years</td>
<td>—</td>
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Material: Morus bombycis Koidz., cv. Kenmochi. Winter buds were slowly frozen to −30°C at the rate of 10°C/day before storage at various temperatures. Winter buds were thawed rapidly in a water bath at 37°C. After sterilization, excised buds were plated on MS medium. Shoot formation of the cryopreserved winter buds was defined as percentage of buds developing normal shoots at 40 days after plating. Approximately 20 buds were treated for each set of duplicates.
Table 2. Rate of shoot formation of winter buds cooled to $-135^\circ$C for 3.5 years.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Shoot formation rate (%)</th>
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<tbody>
<tr>
<td>Araguwa</td>
<td>56.5</td>
</tr>
<tr>
<td>Iikuwa 1</td>
<td>60.0</td>
</tr>
<tr>
<td>Iwate 1</td>
<td>58.8</td>
</tr>
<tr>
<td>Nichirenso</td>
<td>52.6</td>
</tr>
<tr>
<td>Nittaguwa</td>
<td>66.7</td>
</tr>
<tr>
<td>Hayamaruguwa</td>
<td>84.0</td>
</tr>
<tr>
<td>Haruiroguwa</td>
<td>73.7</td>
</tr>
<tr>
<td>Yamanobeso</td>
<td>73.7</td>
</tr>
<tr>
<td>Shinso 2</td>
<td>84.0</td>
</tr>
<tr>
<td>Hokkaiyayo</td>
<td>100.0</td>
</tr>
<tr>
<td>Jinbei 2</td>
<td>95.5</td>
</tr>
<tr>
<td>Sumonwase</td>
<td>54.0</td>
</tr>
<tr>
<td><strong>Av.</strong></td>
<td><strong>71.6±4.7</strong></td>
</tr>
</tbody>
</table>

Winter buds were cooled to $-30^\circ$C at $10^\circ$C/day before a storage at $-135^\circ$C. Winter buds were thawed rapidly in a water bath at $37^\circ$C. After sterilization, the excised buds were plated on MS medium. Shoot formation of cryopreserved winter buds was defined as percent of buds developing normal shoots at 40 days after plating. Approximately 20 buds were treated for each cultivar.

Any form of genetic aberration from the genotype of the germplasm should be viewed with extreme caution. Meristem culture technique is being used not only for clonal propagation, but also for the supply of virus-free plants. Furthermore, the progenies regenerated by *in vitro* culture of meristem have, so far, displayed a genetic stability to a larger extent compared to other methods of *in vitro* plant regeneration (KARTHA, 1985).

Cryopreservation of dormant winter buds provides several advantages as a method of conserving woody plant resources: (1) clonal integrity is maintained; (2) the method is simple, reliable and space-efficient; (3) it requires no expensive and sophisticated cooling apparatus; (4) very long storage is theoretically possible; (5) clonal propagation *in vitro* from cryopreserved materials is easy; (6) it avoids possible toxic effects of chemical cryoprotectants that are required in conventional methods.

In our laboratory, about 420 mulberry cultivars are maintained in the field. Concurrently, winter buds of the same cultivars have been

![Fig. 1 Chromosomes of plants regenerated from winter buds cooled to $-196^\circ$C (left) and $-135^\circ$C (right) for 3.5 years. Material : Kenmochi (2 n = 28). (× 1,000).](image-url)
cryopreserved in a deep freezer at $-135^\circ$C for more than 4 years. This cryogenic method appears to be promising as a routine method for cryopreserving winter buds of woody plants.

References


