Stability of High Cryoprotectability of *Lavandula vera* Cell Lines Selected by Repeating a Freeze-thaw Procedure

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The cryoprotectability of viable *Lavandula vera* cells after repetition of a freeze-thaw procedure one to three times was lowered after continuous subculture on nutrient medium for ten generations (ca. one year) without being subjected to the procedure. However, high cryoprotectability of viable cells after repeating the freeze-thaw procedure four times did not change after the continuous subculture. The frequency of redifferentiation of viable cells after one freeze-thaw procedure was high. These findings suggest that cells with stable high cryoprotectability could be selected by repeated freezing and thawing, and that the formation of shoots and/or roots was induced by the stress resulting from freezing.

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Introduction

Cryostorage of plant cells in LN₂ (liquid nitrogen) is one suitable method to conserve desired potential of cells such as productivity of secondary metabolites and resistance to chemicals, and that the frequency of shoot- and/or root-formation of frozen-thawed cells is high compared to that of unfrozen cells. High cryoprotectable cells have been obtained by repeated freezing and thawing. It has been reported that the high cryoprotectable cells adapt to low temperatures by the accumulation of un-

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saturated fatty acids, an increase in the fluidity of plasma membrane and a lowering of the thermotropic transition point of the membrane, thus surviving the subsequent cryopreservation in LN2.5).

However, some of the desired potential of selected cells such as productivity of secondary metabolites and redifferentiation is lost during long-term subculture without cell selection. This leads to the question of whether the high cryoprotectability of cells selected by repeated freezing and thawing is stable or not. Therefore, we studied the change in cryoprotectability of selected cell lines by long-term subculture without cell selection. Also, we examined the relation between redifferentiation of frozen-thawed cells and cryoprotectability of cells.

Materials and Methods

Callus culture
Cultured green Lavandula vera cells were used. They were subcultured on Linsmaier & Skoog (LS) agar (0.9% w/v) medium8 supplemented with 3% (w/v) sucrose, 10 μM indole-3-butyric acid and 1 μM 6-benzylaminopurine as described previously4).

Selection of cryoprotectable cells by repeating a freeze-thaw procedure
A freezing and thawing procedure was repeated to obtain high cryoprotectable cells, as described previously4). Small cell aggregates (< 1 mm) were cut from cultured green L. vera cells in the logarithmic growth phase (7 to 10 days) using forceps, and suspended in LS liquid culture medium after which the cell suspension was chilled on ice. A mixture of 10% (v/v) dimethylsulfoxide and 20% (w/v) D-glucose (the cryoprotectant solution) was prepared in distilled water, sterilized by filtration and chilled on ice. This ice-chilled cryoprotectant solution was added gradually to an equal volume of the cell suspension over a period of 1 hr, after which the resulting cell suspension was dispensed in sterile polypropylene tubes on ice (0.5 ml settled cells + 0.5 ml of liquid / 2-ml tube). The cell suspension was cooled at 1°C / min to −40°C in a Cryo-Med programmed freezer (Mt. Clemens, Mich.), then transferred into LN2. After 7 to 14 days of storage in LN2, the cell suspension was thawed rapidly by agitation of tube in a 60°C water bath for between 40 to 60 seconds just before the ice in the tube melted completely, then the tube was chilled on ice. Immediately after thawing, 0.5 ml of the thawed cells (settled cell volume) was spread on 20 ml of the semi-solidified LS agar (0.5% w/v) medium in a 9-cm petri dish without washing the cells with liquid culture medium. The spread cells were cultured under the same conditions as described above for up to 3 months. The numbers of colonies formed and those having shoots and/or roots were counted on each petri dish.

Dedifferentiated callus colonies that grew vigorously were chosen from each petri dish. Then the colonies were individually transferred to LS agar (0.9% w/v) medium and cultured under the conditions described above for 30 to 50 days. Cell aggregates that developed from these col-
colonies were transferred to fresh LS agar medium. Cells in the logarithmic growth phase were frozen again, then thawed and cultured according to the same procedure described above.

*Stability of high cryoprotectability and differential potential of cells viable after repeated freeze-thaw procedures*

To evaluate the stability of high cryoprotectability of the cells selected after repeated freezing and thawing procedures, some of the cells were cultured on the LS agar medium continuously for ten generations (ca. one year) without being subjected to selection by freezing and thawing, after which they were subjected to one freezing and thawing procedure. After storage in LN₂, 0.5 ml of the thawed cells was spread on 20 ml of the semi-solidified LS agar medium in a 9-cm petri dish. The spread cells were cultured as described above. The stability of cryoprotectability of these cells was judged from the number of colonies formed per petri dish.

Also, to examine the relation between redifferentiation of frozen-thawed cells and cryoprotectability of cells, the frequency of shoot-and/or root-formation of cells before and after continuous subculture on LS agar medium for ten generations without cell selection by freezing and thawing was determined.

*Results and Discussion*

*Changes of colony formation and redifferentiation of cells by repetition of the freeze-thaw procedure*

The changes in the frequencies of colony formation and redifferentiation of frozen-thawed cells by repeating the freeze-thaw procedure are shown in Fig. 1. Unfrozen control cells formed more than 1,000 colonies per petri dish. After the 1st procedure, <1 % of the freeze-thawed cells formed colonies. The frequency of colony formation increased after repetition of the procedure. The average value (112 col-

![Number of freeze-thaw cycles](image-url)

**Number of freeze-thaw cycles**

Fig. 1. Changes in the frequencies of colony formation (○) and redifferentiation (●) of cultured green *L. vera* cells subjected to repeated freezing and thawing. The number of formed colonies was counted in at least sixty petri dishes for each procedure, and the average number per petri dish is shown. For frequency of redifferentiation, at least one hundred colonies were counted for each procedure, and the percentage of the colonies having shoots and/or roots is shown.
onies / petri dish) after the 8th procedure was about twenty two times higher than that (5 colonies / petri dish) after the 1st procedure.

The frequency of redifferentiation of viable cells after the 1st procedure (62%) was higher than that (5%) of unfrozen control cells. However, frequency decreased with the repetition of the procedure. After the 8th procedure, only 13% of the formed colonies had shoots and/or roots.

These results suggest that the cells viable after repeated freezing and thawing procedures had high cryoprotectability, thus can be assumed to have been less damaged than the cells viable after the 1st procedure. On the other hand, the frequency of shoot-and/or root-formation of viable cells after the 1st procedure was high. However, the frequency of viable cells after repeated freezing and thawing procedures was low. These findings suggest that high frequency of shoot- and/or root-formation of frozen-thawed cells might be due to stress resulting from freezing and thawing, not cell selection by freezing and thawing.

**Stability of high cryoprotectability and differential potential of the cells viable after repeated freeze-thaw procedures**

To evaluate the stability of high cryoprotectability and differential potential of the surviving cells, some of the cells were cultured on LS agar medium continuously for ten generations (ca. one year) without being subjected to selection by freezing and thawing, after which they were subjected to one freezing and thawing procedure, as described in Materials and Methods. The frequency of colony formation of these cells is shown in Fig.2. The frequency of colony formation of viable cells after one to three freeze-thaw procedures was lowered after continuous subculture for ten generations without being subjected to selection by freezing and thawing. However, viable cells after four procedures formed many colonies after continuous subculture as well as before continuous subculture.

Table 1 shows the frequency of redifferentiation of cells before and after continuous subculture for ten generations without subjection to selection by freezing and thawing. The frequency of redifferentiation of viable cells after four procedures before continuous subculture was equal to that after continuous subculture. On the

![Fig. 2. Frequency of colony formation of cells subjected to freezing in LN₂ before (upper) and after (lower) continuous subculture on a nutrient medium for ten generations without selection by freezing and thawing. A, Viable cells after one freeze-thaw procedure; B, Viable cells after two procedures; C, Viable cells after three procedures; D, Viable cells after four procedures. x=average number; n=number of petri dishes.](image-url)
Table 1. The frequency of redifferentiation of colonies formed from cells before and after continuous subculture on a nutrient medium for ten generations without being subjected to selection by freezing and thawing.

<table>
<thead>
<tr>
<th>Repeated number of freeze-thaw procedures</th>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before subculture</td>
</tr>
<tr>
<td>0</td>
<td>5(%)</td>
</tr>
<tr>
<td>1</td>
<td>62</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
</tr>
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At least one hundred colonies were counted for each procedure. The percentages of the colonies having shoots and/or roots are shown.

On the other hand, the frequency of viable cells after one to three procedures decreased to 17-20% after continuous subculture for ten generations.

These results suggest that the high cryoprotectability of viable cells after four procedures was stable even after continuous subculture for ten generations (ca. one year) without selection by freezing and thawing. The frequency of redifferentiation of viable cells after one procedure was lowered after continuous subculture for ten generations. The viable cells after one procedure might show damage from the freezing and thawing more than the viable cells after the repeated procedures (Fig. 1). These findings suggest that the viable cells after one procedure might recover during continuous subculture for ten generations, since the frequency of redifferentiation of the cells after continuous subculture was equal to that of the high cryoprotectable viable cells after four procedures (Table 1). Also the high frequency of redifferentiation of frozen-thawed cells was assumed to be due to stress resulting from freezing and thawing (Fig. 1).

The results here show that cell lines with stably high cryoprotectability could be selected by repeated cell selection, and that formation of shoots and/or roots is induced by the stress resulting from freezing in LN₂.

References


