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Cryopreservation of Gametophytes of *Laminaria japonica* (Phaeophyta) Using Encapsulation-Dehydration with Two-Step Cooling Method

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Abstract Gametophytes of *Laminaria japonica* were cryopreserved in liquid nitrogen using encapsulation-dehydration with two-step cooling method. Gametophytes cultured at 10℃ and under continuous irradiance of 30 μmol m^{-2} s^{-1} for 3 weeks were encapsulated in calcium alginate beads. The beads were dehydrated in 0.4 molL^{-1} sucrose prepared with seawater for 6 h, desiccated in an incubator set at 10℃ and 70% relative humidity for 4 h, pre-frozen at either −40℃ or −60℃ for 30 min, and stored in liquid nitrogen for >24 h. As high as 43% of survival rate was observed when gametophytes were thawed by placing the beads in 40℃ seawater and re-hydrated in 0.05 molL^{-1} citrate sodium prepared using 30‰ NaCl 7 d later. More cells of male gametophytes survived the whole procedure in comparison with female gametophytes. The cells of gametophytes surviving the preservation were able to grow asexually and produce morphologically normal sporophytes.

Key words *Laminaria japonica*; gametophyte; encapsulation-dehydration with two-step cooling; cryopreservation

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1 Introduction

Laminaria japonica, an alga with high economic value, was introduced into China for cultivation in 1927. With the innovation and implementation of a floating raft culturing method, commercial cultivation of *L. japonica* came into being progressively from 1952 (Tseng, 1981). At present, about 900 000 metric tons of air-dried kelp and 13 000 metric tons of algin are produced each year in China (Tseng, 2001). In the 1970s, the gametophyte of *L. japonica* was cloned successfully. In this way, many strains can be preserved indoors and applied to breeding (Fang and Dai, 1980). Although gametophyte cloning makes the long-term preservation of the gametophyte of *L. japonica* feasible, the maintenance of many strains requiring both more labor and space and the risks of being contaminated by microbes or being intermixed with some microalge are disadvantageous. Genetic variation may also occur in gametophyte cloning.

Cryopreservation is a reliable technique ensuring the long-term genetic stability of preserved organisms (Grout, 1995). It has been successfully applied to the preservations of animals, plants and microalgae. In recent years, the cryopreservations of macroalgae have been increased (Kuwano and Saga, 2000; Wang *et al*., 2000; Crutchfield *et al*., 1999; Day *et al*., 1998; Ginsburger-Vogel *et al*., 1992; Arbault *et al*., 1990). As for *L. japonica*, only Kuwano *et al.* (2004) reported the cryopreservation using a two-step cooling procedure, and a survival rate of about 70% was achieved. However, the use of toxic cryoprotectants was necessary in this two-step cooling procedure. In addition, the growth and development of thawed gametophytes were not assayed in the previous cryopreservation of *L. japonica*.

Another technique, encapsulation-dehydration, was recently employed successfully in the cryopreservation of both microalgae (Wang *et al*., 2005a, b; Li *et al*., 2003; Day *et al*., 2000; Hirata *et al*., 1996) and macroalgae (Wang *et al*., 2000; Vigneron *et al*., 1997). This technique integrated the dehydration of encapsulated algal cells by means of air drying with immersion and storage in liquid nitrogen, which was independent of toxic cryoprotectants and expensive programmable freezer. However, the application of this method to the species of *Laminaria* was scarce, only *L. digitata* (Vigneron *et al*., 1997) having been cryopreserved in this way with a survival rate of 25%–75%. The effectiveness of cooling methods has been found to vary with the species or the cell types

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(Kuwano and Saga, 2000; Taylor and Fletcher, 1999), and except for the majority of algal species, there has been no successful case at all. In addition, controlled freezing at the first step adopted by Vigneron *et al*. (Vigneron *et al*., 1997) seemed inconvenient for application.

The work reported here is a systematic examination of the various requirements for the cryopreservation of the gametophytes of *L. japonica* using encapsulation-dehydration with a two-step cooling procedure. The growth and development of thawed gametophytes were also investigated.

2 Materials and Methods

2.1 Gametophytes

One sexually mature sporophyte of *L. japonica* was collected and cleaned. The blade with sporangia was cut into pieces, about 10 cm^2 in size, and allowed to release spores in sterilized seawater at 8℃. After being filtrated through a strainer of 30 μm aperture, the spore suspension was poured onto a staining dish containing glass slides. Most spores adhered to glass slides in two hours. The slides then were transferred into seawater supplemented with 3×10^{-4} $molL^{-1}$ N and 2×10^{-5} $molL^{-1}$ P and cultured at 10℃ under the continuous irradiance of 30 $\mu mol\,m^{-2}\,s^{-1}$. Seawater was replaced once a week. The ga- metophyte clones formed in one week were cultured under above conditions or at 5℃ and under irradiance of 10$\mu mol\,m^{-2}\,s^{-1}$ with the rhythm of 10 h light and 14 h dark.

2.2 Encapsulation of Gametophyte

Gametophytes in length of 200−300 μm were encapsulated using the method of Hirata *et al*. (1996). In brief, gametophytes were mixed with 0.03 $kg\,L^{-1}$ alginate sodium, and then dropped into 0.1 $molL^{-1}$ $CaCl_2$ prepared with 0.03 $kg\,L^{-1}$ NaCl using a sterile pipette. The beads about 3.5 mm in diameter formed in 30 min were then transferred onto filter paper to remove the surface liquid.

2.3 Dehydration of Gametophyte-containing Beads

Beads were dehydrated in 0.4 $molL^{-1}$ sucrose prepared using seawater for 6 h (Vigneron *et al*., 1997) and then desiccated either in a Petri dish placed in an incubator set at 10 ℃ and 70% relative humidity; or in a laminar flow cabinet set at 16 ℃ and 40% relative humidity (Vigneron *et al*., 1997), or above silica gel in Petri dishes with cover placed in an electric thermostatic drying oven set at 19 ℃ (Hatanaka *et al*., 1994).

2.4 Determination of Water Content and Dehydration Rate of Beads

The weight of beads newly prepared was recorded as fresh weight (FW). The weight of beads after dehydration with 0.4 $molL^{-1}$ sucrose and then desiccation with different methods was regarded as the post-desiccation weight (PDW). The weight of beads after being dried to constant weight was considered as dry weight (DW). Water content of beads WC (%) = (PDW−DW) / FW × 100%. Dehydration rate = (WC_i − WC_j) / T, where WC_i is the water content at hour *i*, WC_j is the water content at hour *j* and T is the time between two observations in hour.

2.5 Freezing, Thawing and Re-hydrating

The desiccated beads were dispensed into cryotubes, 5−7 beads each. The cryotubes were transferred to freezer set at −20 ℃, −40 ℃, −60 ℃ and −80 ℃, respectively. After being held there for 30 min, 60 min and 90 min, respectively, the cryotubes were immersed in liquid nitrogen and stored for >24 h. As controls, a few cryotubes were also immersed in liquid nitrogen without pre-freezing. Beads were thawed by either plunging the cryotubes into a water bath pre-warmed to 40℃ or being placed nakedly in seawater pre-warmed to 40℃ until the beads melted completely. Gametophytes were re-hydrated at 10 ℃ in 0.05 $molL^{-1}$ citrate sodium prepared using 0.03 $Kg\,L^{-1}$ NaCl immediately or after being stored at 4 ℃ for 7 d, and filtrated out using a strainer of 30 μm aperture.

2.6 Survival Rate Assaying

Re-hydrated gametophytes were cultured in enriched seawater at 10 ℃ and under continuous irradiance of 30 $\mu mol\,m^{-2}\,s^{-1}$. The morphological changes of gametophytes were observed in one week. The survival rate of gametophytes was expressed as the ratio of the number of the cells with brown color (living) to the total. More than 200 cells were checked for each container.

2.7 Data Processing

The mean and standard deviation were calculated for all observations. These observations each were carried out three times, two replicates each time. The origin 7.0 software (http://www.originlab.com) was used to process data.

3 Results

3.1 Effect of Desiccation Method and Water Content of Beads

The fastest desiccation was achieved in a laminar flow cabinet, where the water content of beads was reduced to about 40% in 3 h, and dehydration rate was 15%−20% h^{-1}. The slowest desiccation was observed above silica gel, where the water content of beads was not reduced to 40% until 10 h, and dehydration rate was 5%−11% h^{-1}. Water content of beads in an incubator set at 10℃ and 70% relative humidity was reduced to about 40% in 4 h, and the dehydration rate was 10%−15% h^{-1} (Fig.1).

When the water content of beads was >70% or <20%, almost all cells died after thaw. If the water content was >20% but < 70%, post-thaw survival rate of gameto-

phytes was different among 3 desiccation methods. Relative high survival rate was observed when beads were desiccated in an incubator. For all methods, the best post-thaw survival rate was observed when the water content of beads was 40%. Desiccation in an incubator was found to be the most effective (Fig.2).

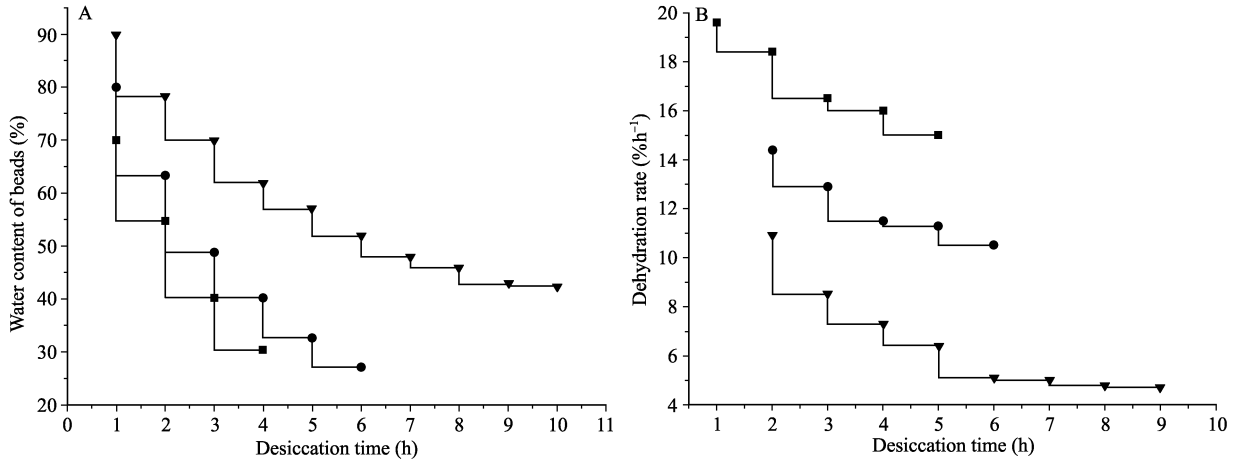

Fig.1 Water contents of beads (A) and dehydration rates (B) obtained using different methods. Beads containing gametophytes were dehydrated in 0.4 M sucrose for 6 h and then desiccated either in a laminar flow cabinet set at 16 ℃ and 40% relative humidity (■), or in an incubator set at 10 ℃ and 70% relative humidity (●), or above silica gel in a drying oven set at 19℃ (▼).

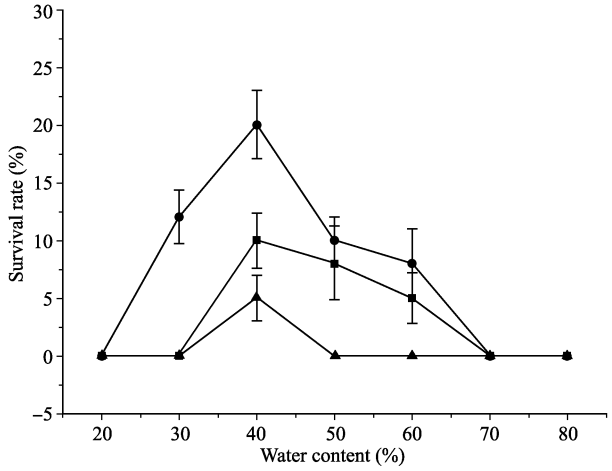

Fig.2 Post-thaw survival rates of gametophytes in beads dehydrated in 0.4 M sucrose for 6 h, desiccated either in a laminar flow cabinet set at 16℃ and 40% relative humidity (▲), or in an incubator set at 10℃ and 70% relative humidity (●), or above silica gel in a drying oven set at 19℃ (■), and then immersed in liquid nitrogen, stored there for 24 h and thawed by plunging the cryotube into water bath pre-warmed to 40℃.

3.2 Effect of Two-step Cooling

Fig.3 illustrates the post-thaw survival rate of the gametophytes in beads dehydrated in 0.4 molL^{-1} sucrose for 6 h and then desiccated in an incubator set at 10℃ and 70% relative humidity, prefrozen and stored in liquid nitrogen for >24 h. Post-thaw survival rate of gametophytes was less than 17% when beads were immersed in liquid nitrogen without pre-freezing. Pre-freezing at −40 ℃ and −60 ℃ for 30–60 min caused relatively high post-thaw survival rate of the gametophytes. The best preserving efficiency was observed when the beads were desiccated for 4 h and pre-frozen at either −40 ℃ or −60 ℃ for 30 min.

3.3 Growth and Development of Thawed Gametophytes

Living cells of gametophytes re-hydrated and cultured at 10 ℃ and under continuous irradiance of 30 μmol m^{-2}s^{-1} for 7 d showed brown color and started to divide, while the dead ones lost their cytochrome and became vacant (Fig.4A). One month later, living cells grew into new gametophyte clones, forming protonema about 1mm in diameter (Fig.4B). When thawed gametophytes were cultured at 10 ℃ and under the irradiance of 30 μmol m^{-2} s^{-1} following the rhythm of 12 h light and 12 h dark, living cells recovered in a similar way as those under the continuous irradiance within 7 d. In two weeks, a few living cells of gametophytes started to develop and produce sporophytes (Fig.4C). In three weeks, about 50% of the female gametophytes formed sporophytes (Fig.4D). Morphological difference was not detected between sporophytes generated from thawed gametophytes and those from the normal gametophytes.

3.4 Effect of Other Factors

Besides desiccation and pre-freezing, post-thaw survival rate of gametophytes was also influenced by the culturing condition of gametophytes prior to encapsulation and the sex of gametophyte, but not by thawing method and re-hydrating method (Table 1). Two culturing conditions (A and B) were widely adopted in germplasm preservation and vegetative amplification of gametophyte clones. It was found that survival rates obtained using these two conditions were significantly different ($p < 0.01$); about 10% higher survival rate was observed at condition B than at condition A. Although no significant differences were found between the two

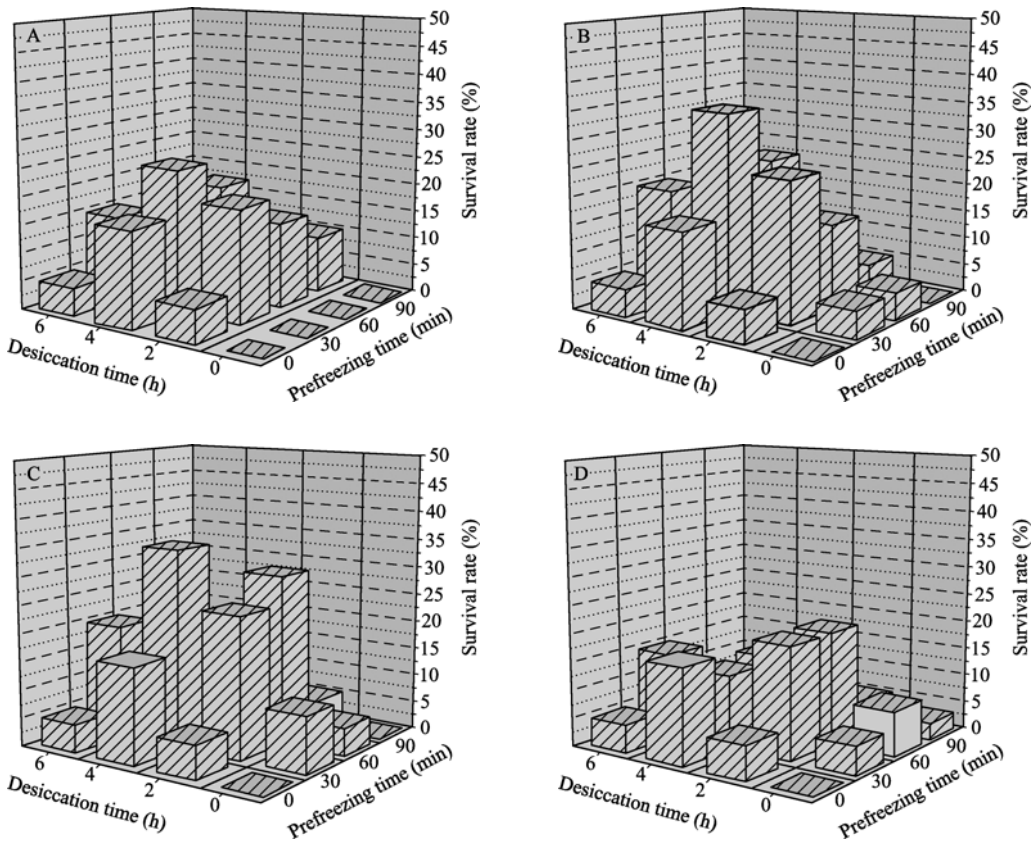

Fig.3 Post-thaw survival rates of gametophytes in beads dehydrated, desiccated in an incubator set at 10℃ and 70% relative humidity for different times and prefrozen at −20℃ (A), −40℃ (B), −60℃ (C) and −80℃ (D) for different time prior to immersion in liquid nitrogen. After being stored for 24 h, beads were thawed by plunging the cryotubes into water bath pre-warmed to 40℃.

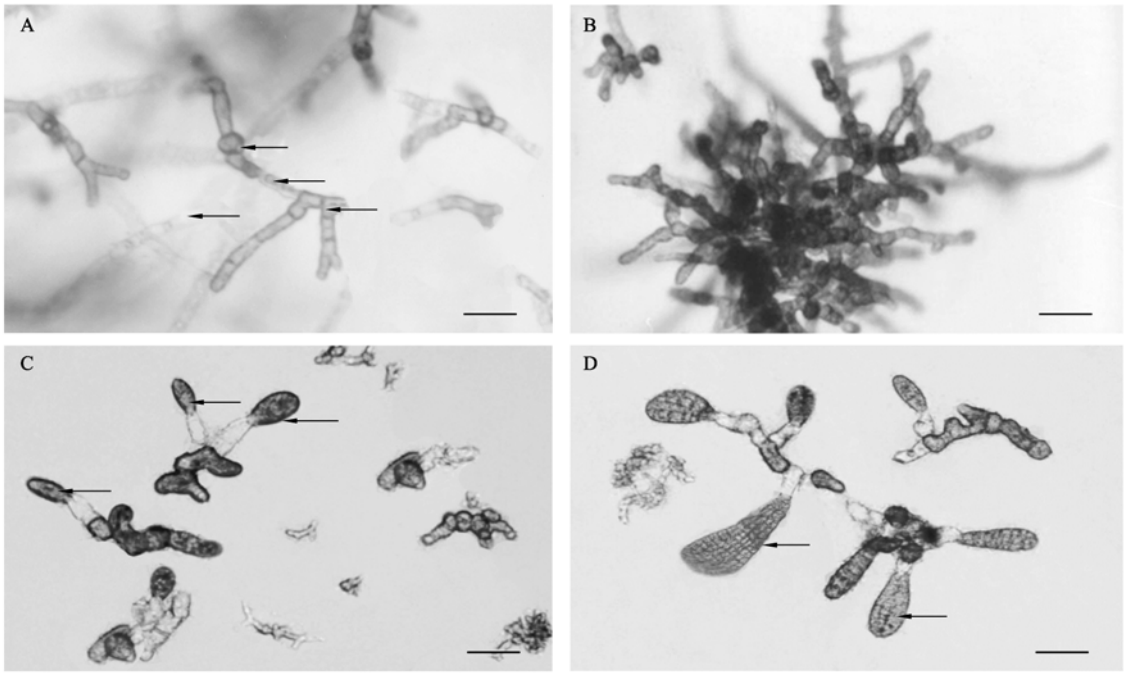

Fig.4 Cryopreserved gametophytes growing and developing after being thawed. Living cells (coarse arrow) and the dead (fine arrow) observed on the 7th day of cultivation (A); newly formed gametophytes from living cells (B); sporophytes (fine arrow) generated from newly formed gametophytes observed on the 15th day of cultivation (C); and sporophytes (fine arrows) observed on the 21st day of cultivation (D). Scale bars: A and B, 20 μm; C and D, 100 μm.

Table 1 Effects of preculturing condition of gametophytes, thawing method and re-hydrating method on the post-thaw survival rate of cells

Culturing conditions	Thawing method	Re-hydrating method	Survival rate (mean ± SD)†	Male/female††
A	I	(1)	22.3 ± 3.4	1.27:1
A	II	(1)	27.2 ± 4.3	1.50:1
A	I	(2)	28.4 ± 2.3	1.88:1
A	II	(2)	34.5 ± 4.5	1.63:1
B	I	(1)	33.3 ± 3.7	2.45:1
B	II	(1)	39.6 ± 6.8	1.86:1
B	I	(2)	39.0 ± 6.8	1.38:1
B	II	(2)	43.2 ± 3.7	2.33:1

Notes: A: irradiance of 10 μmol $m^{-2}s^{-1}$ with the rhythm of 10 h light and 14 h dark, 5 ℃; B: continuous irradiance of 30 μmol $m^{-2}s^{-1}$, 10 ℃; I, plunging cryotube into water bath; II, placing beads in seawater; (1), re-hydrating immediately after thawing; (2), re-hydrating on the seventh day after thawing. †Data in percentage. ††Living gametophytes.

Beads were dehydrated in 0.4 M sucrose for 6 h, desiccated in an incubator set at 10 ℃ and 70% relative humidity for 4 h, and pre-frozen at −40 ℃ for 30 min prior to immersion in liquid nitrogen and stored there for more than 24 h.

Difference of average survival rate between A and B is significant ($p < 0.01$), and those between I and II and (1) and (2) are not.

thawing methods and two re-hydrating time, the highest post-thaw survival rate of gametophytes pre-cultured under condition B for 3 weeks (43%) was observed when beads were thawed by placing them in seawater pre-warmed to 40℃ and re-hydrated one week later. In addition, skew ratio of male to female gametophytes surviving the cryopreservation was observed. The number of viable male gametophytes after thawing was significantly more than female ones under various conditions (Table 1).

4 Discussion

Many protocols are currently available for the cryopreservation of high plants, such as standard two-step cooling, dehydration-desiccation, vitrification, encapsulation-dehydration and encapsulation-vitrification (Yi and Deng, 1999). Among them, two-step cooling and encapsulation-dehydration are adopted for algal preservation more often than others. Reports about the application of encapsulation-dehydration for algal preservation have been increasing recently (Wang *et al*., 2005a, b; Li *et al*., 2003; Wang *et al*., 2000); however, the application of encapsulation-dehydration with two-step cooling for algae is still limited, though this procedure was considered as a promising one for macroalgae (Day *et al*., 2000). Day *et al.* (2000) and Vigneron *et al*. (1997) applied this technique to the preservation of *Euglena gracilis* and *L. digitata*, respectively. The present study is the first report about the cryopreservation of *L. japonica* using encapsulation-dehydration with two-step cooling. About 43% of post-thaw survival rate was obtained in present study, which was lower than that reported previously using two-step cooling procedure. However, our method does not depend on toxic cryoprotectants. This avoids the toxic effects of ethylene glycol and praline used in the previous works (Kuwano *et al*., 2004).

In order to make the preservation of cells by encapsulation-dehydration successful, the cells must be dehydrated to a water content at which the cytosol is concentrated appropriately so that the cooling below the thermodynamic freezing point without homogeneous ice nucleation is allowed (Stillinger, 1995). Beads that are insufficiently dehydrated will encounter lethal ice nucleation during freezing and thawing (Day *et al*., 2000). In the present study, almost all cells died when encapsulated gametophytes were plunged into liquid nitrogen without desiccation (Fig.3). The post-thaw survival rate of gametophytes directly plunged into liquid nitrogen was not improved distinctly by dehydration and desiccation. However, all the post-thaw survival rates of gametophytes cooled using two-step cooling procedure after dehydration were increased to some extent (Fig.3). Among them, the highest survival rate was increased to 35% from less than 17% (Figs.3B, C). Therefore, the two-step cooling procedure prior to immersion in liquid nitrogen is thought to make the gametophytes dehydrate deeper, and prevents ice-crystal from forming. This technique is thought to be more suitable for those species that are highly sensitive to desiccation. The effect of encapsulation-dehydration with two-step cooling on the post-thaw survival rate varies among species. Some species have the same viability after being preserved using encapsulation-dehydration and encapsulation-dehydration with two-step cooling (Scottez *et al*., 1992), while the others show the different viabilities after being preserved using these two methods (Yi and Deng, 1999).

Dehydration is a key step of the cryopreservation of high plants using encapsulation-dehydration with or without two-step cooling (Scottez *et al*., 1992). However, the effect of dehydration rate on the cryopreservation has received less attention. Such effect was reported only in *Porphyra haitanensis* (Wang *et al*., 2000), *Undaria pinnatifida* (Wang *et al.,* 2005a, b) and a few microalgae

(Hirata *et al.*, 1996). We found that both water content of beads and dehydration rate influenced the post-thaw survival rate of gametophytes of *L. japonica*. Vigneron *et al*. (1997) found that *L. digitata* was better cryopreserved at low dehydration rate. However, this trend was not found in the cryopreservation of *L. japonica* (data not shown). Instead, we found that an appropriate dehydration rate is crucial for the cryopreservation of *L. japonica*. If the rate is too high, intracellular freezing may occur because of an unbalanced dehydration between cells and bead (Hirata *et al*., 1996), and if the rate is too low, the damage caused by desiccation and higher temperature can not be avoided.

Our result showed that male gametophytes are more tolerant to the whole procedure of cryopreservation than female gametophytes. This was also observed in the other species of Laminariales, such as *L. longissima*, *Kjellmanniella crassifolia*, *Ecklonia stolonifera* and *E. kurome* (Kuwano *et al*., 2004). Difference of cell configuration, structure and physiological characteristics between female and male gametophytes may cause the difference of their tolerance to cryopreservation. Grout (1995) suggested that large cells lose less water than small cells do during freezing, making smaller cells less prone to intracellular ice formation during freezing. The male gametophyte cells are smaller than the female gametophyte cells, and therefore, may be more tolerant to the cryopreservation than the female gametophytes.

Less attention has been paid to the improvement of post-thaw viability by inducing freeze-tolerance in macroalgae than in microalgae (Taylor and Fletcher, 1999). The induction methods of freeze-tolerance reported previously in microalgae include low temperature acclimation, choose of culture age, and alteration of salinity, nutrient levels and water content prior to freezing (Fenwick and Day, 1992; Hirata *et al*., 1996; Mortain-Bertrand *et al*., 1996; Cañavate and Lubian, 1997). Although the optimum induction appears to vary with the cell types, most freezing acclimations were induced by the reduction of metabolic rate, as Ben-Amotz and Gilboa (1980) suggested. In the present study, two kinds of culture conditions of gametophytes, serial subculture (Condition A, commonly used for germplasm preservation) and vegetative amplification (Condition B), were employed for the examination of the effect of pre-culture condition on the post-thaw survival. We found that pre-culture condition consisting of light intensity, photoperiod and temperature played an important role in achieving high post-thaw survival of cryopreserved gametophytes (Table 1). Besides, Condition B which is favorable for the vegetative growth of *L. japonica* gametophyte significantly improved the post-thaw survival rate. Algae grown under unfavorable conditions may develop the intrinsic chemical and physical properties which endow them with tolerance to freezing (Taylor and Fletcher, 1999). Our findings are not in accordance with this conclusion, but similar with those obtained in some members of microalgae in genus *Tetraselmis* (Day and Fenwick, 1993). Cells of these microalgae grown at a sub-optimal temperature of 10°C had lower survival rate than those grown at ambient temperatures ranging from 15 to 25°C. The mechanism of freeze-tolerance is not clear (Taylor and Fletcher, 1999), and the ambient factors involved in the metabolic vigor of *Laminaria* gametophytes are multitudinous and interactive. Therefore, the effect of pre-culture conditions on the post-thaw survival rate of *L. japonica* gametophytes should be studied further. In addition, supplemented abscisic acid in pre-culture medium can improve the desiccation tolerance of a few plants (Li *et al*., 1999). It is worthy of trying to use abscisic acid in the pre-culture of the gametophytes of *L. japonica* in order to improve the desiccation tolerance.

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