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Effect of Dimethylsulfoxide on *in vitro* Storage of Wasabi Meristems at Low Temperature

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Abstract

Long-term storage of *in vitro*-grown wasabi (*Wasabia japonica* MATSUMURA) meristems at low temperature using dimethylsulfoxide (DMSO) were investigated. Meristems were plated on solidified 1/2 MS medium supplemented with 3 % sucrose and 1 % DMSO for a day, and then they were successfully stored at − 5 ℃ for 24 months. After the storage, meristems were recultured on solidified 1/2 MS medium supplemented with 3 % sucrose and 0.1 mg l^{-1} 6-benzyladenine (BA). The survival rate was about 60 %. Although many meristems appeared water-soaked after storage, most recovered to normal shoots.

A conventional method of cultivation known as a field gene bank has been used for the maintenance of wasabi germplasm. The conventional method has some disadvantages such as a high cost for long-term maintenance, and the risk of genetic loss due to unpredictable weather conditions and diseases. To overcome these problems, preservation of wasabi germplasm in test tubes at 20 ℃ (Yamada *et al.*, 1992) and cryopreservation at super low temperatures such as − 196 ℃ (Matsumoto *et al.*, 1994) were developed. The use of these methods provides advantages such as a high capacity to store germplasm in a small space, and elimination of the risk of unfavorable natural conditions. However, the cost of *in vitro* subcultures and possibility of somaclonal variation are currently problems of preservation in test tubes. In the cryopreservation method for inducing the high survival after storage, some complicated treatments such as preculture of sucrose enriched medium, cryoprotection and dehydration are necessary processes before rapid cooling to super low temperature. Here, the authors have investigated a novel method of *in vitro* storage of wasabi meristems at low temperature using DMSO.

In vitro-cultured plantlets of wasabi were used in this study. Stock cultures of wasabi were maintained on modified Murashige and Skoog (1962) basal medium (half strength of KNO_3 and NH_4NO_3, termed 1/2 MS) containing 0.1 mg l^{-1} BA, 3 % (w/v) sucrose and 0.2 % (w/v) gellan gum at pH 5.8 (Yamada *et al.*, 1992). Meristems, approximately 1 mm in diameter, were dissected from stock cultures. Twenty-five meristems were plated onto a plastic petri dish containing 1/2 MS medium supplemented with 1 % (v/v) DMSO, 3 % sucrose and 0.2 % gellan gum at pH 5.8, and cultured at 20 ℃ in a 16 h daylight of 52 μmol s^{-1} m^{-2} of white fluorescent light for a day. Then they were stored at − 5 ℃ for 2 to 24 months. After storage, meristems were recultured by transferring to solidified 1/2 MS medium supplemented with 0.1mg l^{-1} BA and 3 % sucrose.

In order to increase the survival of wasabi meristems stored at low temperature, the optimum concentration of DMSO was determined as a cryoprotectant with solidified 1/2 MS medium containing 0.1mg l^{-1} BA and 3 % sucrose (conservation medium for wasabi meristems, termed WC medium). The high level of survival (approx. 95 %) was obtained by adding 0.1 and 1 % of DMSO in WC medium after storage for 1 month at − 5 ℃ (**Fig. 1**). Addition of 1 % DMSO was observed to control the elongation of wasabi meristems during the storage compared to 0.1 % DMSO. The effects of other cryoprotectants, ethylene glycol (EG) and glycerol at 1 % (v/v), were investigated for storage at − 5 ℃ for 2 months (**Table 1**). When using WC medium with or without these two cryoprotectants, the survival rates of wasabi meristems were negligible. For all experiments requiring the storage of wasabi meristems at low temperature, a 1 % of DMSO was determined as the optimal concentration.

Shoot elongation during storage at − 3 to − 7 ℃ for 6 months was investigated. As shown in **Table 2**, there was no difference in the survival rate of wasabi meristems stored at − 3 ℃ and − 5 ℃ for a 6-month period. However, shoot elongation was slightly impaired for wasabi meristems stored at − 5

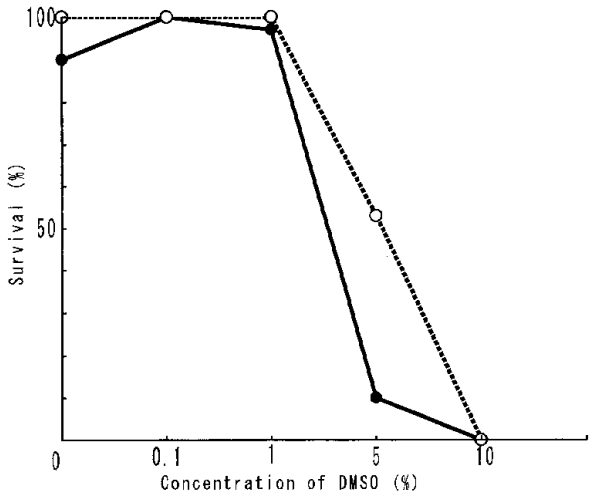

Fig. 1 Effect of DMSO concentration on survival of wasabi meristems stored for 1 month at − 3 and − 5 ℃.
Meristems were stored on solidified 1/2 MS medium supplemented with several concentration of DMSO at − 3 ℃ (○) or − 5 ℃ (●) in a dark for 1 month. Twenty-five meristems were tested for each of two replicates.

Table 1. Effect of cryoprotectants on survival of wasabi meristems stored for 2 months at − 5 ℃.

Cryoprotectant	Survival rate (%)
Control (without cryoprotectant)	0
DMSO	100
Glycerol	12.5
Ethylene glycol	0

Meristems were stored on solidified 1/2 MS medium supplemented with 3 % sucrose and each cryoprotectant at 1 % under dark for 2 months. About twenty-five meristems were tested for each of two replicates.

Table 2. Shoots elongation of wasabi meristems stored at low temperature for 6 months.

Temp. of storage	Degrees of shoot elongation (%)			Survival (%)
	3 >	10-3	> 10 mm	
− 3 ℃	49	30	13	92
− 5 ℃	44	42	6	92
− 7 ℃	0	0	0	0

Meristems were stored on solidified 1/2 MS medium supplemented with 3 % sucrose and 1 % DMSO in a dark for 6 months. Twenty-five meristems were tested for each of 4 replicates.

℃ when compared to − 3 ℃ stored meristems. No meristem stored at − 7 ℃ survived after transfer to reculturing medium. It seemed that the meristems all died due to freezing.

Table 3. Effect of light condition on survival of wasabi meristems stored for 10 and 24 months at − 5 ℃.

Light condition during storage	Survival rate (%) for	
	10 months	24 months
Light	74	64
Dark	86*	62

Meristems were stored on solidified 1/2 MS medium supplemented with 3 % sucrose and 1 % DMSO for 10 and 24 months. Twenty-five meristems were tested for each of two replicates. *Significant at 5 % level by t-test.

The effect of light conditioning during storage at − 5 ℃ was investigated. After 10 months of storage at − 5 ℃, samples in dark-condition had a higher survival rate than that in light-conditioned samples (**Table 3**). After 24 months of storage, survival rates for light and dark-conditioned samples were almost the same level (approx. 60 %). Roxas *et al.* (1995) and Oka *et al.* (1997) demonstrated the ability to preserve dark-conditioned apical buds of *Chrysanthemum* and mulberry bud clusters at 5 ℃, respectively. On the other hand, Hosoki (1989) and Fukai (1989) reported that continuous light was required for the conservation of shoot tips of *Chrysanthemum* at 10 ℃. Terahashi (1992) reported that photosynthesis was seriously affected in plants in illumination at low temperature. These reports suggest that a dark-conditioning treatment may be suitable for *in vitro* storage below 5 ℃ for plant germplasm. Many meristems elongated during storage for 24 months at − 5 ℃ (approx. 10 mm) and appeared water-soaked. However, after transferring to WC medium, most of the meristems recovered normal shoots.

The effectiveness of DMSO as a cryoprotectant for storage at low temperature was first demonstrated by Lovelock *et al.* (1959) using animal cells. Since then, long-term storage in liquid nitrogen, using DMSO was also found effective for fish cells (Wolf *et al.*, 1962) and animal embryos (Tsunoda *et al.*, 1982). In plants, long-term preservation of shoot tips was reported using DMSO as a cryoprotectant (Seibert, 1976; Kartha *et al.*, 1980; Liu *et al.*, 1998). Additionally, plant cells cooled rapidly by immersion into liquid nitrogen (− 196 ℃) formed a metastable glassy state without undergoing lethal intracellular freezing (Sakai *et al.*, 1967; Fahy *et al.*, 1984). Although the toxicity of DMSO for plant cells was pointed out in a previous report (Withers,

1985), it did not occurred during storage in liquid nitrogen due to glass formation. A glass is exceedingly viscous and stopped all chemical reactions that require molecular diffusion (Burke, 1986). On the other hand, wasabi meristems were stored at − 5 ℃ in this study. At this temperature, cell functions slowed down but do not stop. In fact, the toxicity of DMSO against wasabi meristems was observed in the concentration more than 5 % during storage at − 5 ℃. The survival rate after 2 months of storage at − 5 ℃ was significantly decreased (below 10 %). However, the survival rate was increased to approximately 90 %, when the DMSO concentration was 1 %. These results indicate that DMSO is not toxic to wasabi meristems at the concentration less than 1 %.

We also investigated the storage of lily meristems using the same procedure described above. However, few meristems survived 24 months after storage at − 5℃. Thus, it seems that the effect of DMSO as a cryoprotectant for low temperature storage is species specific. Moreover, the possibility of variation on plants stored at low temperature has been pointed out (Fukai, 1992). No morphological abnormalities were observed in regenerated wasabi plants after storage at − 5 ℃ for 24 months in this study. However, further study is necessary to confirm the genetical stability of germplasm stored at low temperature.

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