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Cryopreservation of *in vitro*-grown shoot tips of taro (*Colocasia esculenta* (L.) Schott) by vitrification. 1. Investigation of basic conditions of the vitrification procedure

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Abstract *In vitro*-grown shoot tips of taro (*Colocasia esculenta* (L.) Schott.) were successfully cryopreserved by vitrification. Excised shoot tips precultured on solidified MS supplemented with 0.3 *M* sucrose and maintained under a 16 h phtoperiod at 25°C for 16 h were loaded with a mixture of 2 *M* glycerol plus 0.4 *M* sucrose for 20 min at 25°C. The shoot tips were then sufficiently dehydrated with a highly concentrated vitrification solution (PVS2) for 20 min at 25°C prior to immersion into liquid nitrogen. Successfully vitrified and warmed shoot tips resumed growth within 7 days and developed shoots directly without intermediate callus formation. The average rate of shoot recovery amounted to around 80%, and the vitrification protocol appeared to be very promising for the cryopreservation of taro germplasm.

Key words Shoot tips · Cryopreservation · Vitrification · Taro [*Colocasia esculenta* (L.) Schott.] · Tropical crops

Abbreviations *DMSO* Dimethylsulfoxide · *EG* ethylene glycol · *LN* liquid nitrogen · *MS* Murashige & Skoog medium (1962) · *TDZ* thidiazuron

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Introduction

Taro [*Colocasia esculenta* (L.) Schott] is one of the common and important tuber crops grown throughout the humid tropics and subtropics. This crop provides edible starchy corms, and its young leaves are also used as vegetables. Taro is usually propagated by the leaf-bearing top of mature corms, small lateral tubers or suckers, and genetic resources of this species are mainly conserved vegetatively in field gene banks, which is costly and laborious. There is a growing need for stable long-term *in vitro* storage of the germplasm collections of taro as well as those of other vegetatively propagated tropical crops.

Through the recent remarkable progress in cryobiological studies of plant materials, cryopreservation has been recognized as a practical and efficient tool for long-term storage of vegetatively propagated plant germplasm with minimum space and maintenance requirements. Potentially valuable cryogenic procedures such as vitrification (Sakai *et al.* 1990; Langis *et al.* 1990; Yamada *et al.* 1991), encapsulation/vitrification (Matsumoto *et al.* 1995b) and encapsulation/dehydration (Dereuddre *et al.* 1990; Fabre and Dereuddre 1990) have been developed, and the number of species that can be cryopreserved has increased sharply in recent years. Among different strategies for successful cryopreservation, vitrification has simplified explant handling and secured the survival of shoot tips of species where other cryopreservation techniques had failed (Towill and Jarret 1992).

Cryopreservation of tropical or sub-tropical species has been less extensively investigated than that of cold hardy species. Although cells and somatic embryos of about 30 tropical plant species have been well-preserved in LN, information on the effective cryopreservation of meristems is still very limited (Engelmann 1991).

Embryogenic callus of taro was successfully cryopreserved by slow freezing with 75% survival rate without loosing its regeneration ability (Shimonishi *et al.* 1993). However, for germplasm preservation, meristems are more desirable than embryogenic callus since they avoid the pro-

duction of variants commonly associated with shoot regeneration from callus. To our knowledge, there are few or no reports on the cryopreservation of meristems of taro except that reported by us (Takagi *et al.* 1994). In that report, vitrified axillary buds of taro survived after exposure to LN, but the survival rate remained low and further studies were required.

The study described here, therefore, was aimed at developing a more simple and reliable protocol for the cryopreservation of *in vitro*-grown shoot tips of taro by vitrification.

Materials and methods

Plant materials

In vitro stock plants of taro [*Colocasia esculenta* (L.) Schott] were obtained by culturing 10-mm-long shoot tips, isolated from sterilized field-grown cormels, in liquid MS supplemented with 0.1 mg l^{-1} TDZ and 3% sucrose at pH 5.8 in a 100 ml mayonnaise bottle. Each bottle containing one shoot tip with 10 ml medium was shaken at 100 rpm on a reciprocal shaker .

Axillary offshoots from the *in vitro*-grown stock plants were micropropagated on MS containing 3% sucrose and 0.2% gellan gum (Wako Pure Chemical Industries, Japan) at pH 5.8. The offshoots constituted the stock materials used for subsequent investigations and were subcultured at 2-month intervals. All of the cultures were incubated using cool-white fluorescent lights (ca. 92 μE m^{-2} s^{-1}), under a 16-h photoperiod at 25°C.

To optimize the cryogenic procedures, we used *C. esculenta* var *antiquorum* cv. 'Eguimo' in the present study, and the developed protocol was applied to a second cultivar, 'Dodare'.

Different sizes of dissected shoot tips

Shoot tips dissected in different sizes and axillary buds were compared for their suitability as materials for vitrification. Apical shoot tips were dissected from *in vitro*-grown plants into the following sizes: (1) approximately 2 mm long containing apical dome and 2–3 leaf primordia (referred to as 2 mm-shoot tips hereafter), and (2) 0.5 to 0.8 mm long containing apical dome and 1–2 leaf primordia (referred to as 0.8 mm-shoot tips hereafter). Axillary buds were induced by culturing slices of young corms 5 mm-thick from 2 month-old *in vitro* plants on MS supplemented with 3% sucrose and 0.2% gellan gum for 1 week. The length of the dissected axillary buds, containing apical dome and 3–4 immature leaves, was 1 to 1.5 mm.

Vitrification procedure

Dissected plant materials were immediately used or precultured on solidified MS containing different concentrations of sucrose under dark conditions at 25°C for 16 h.

The precultured explants were loaded with various cryoprotective solutions in petri dishes (30 mm in diameter) containing 5 ml of the loading solutions at 25°C. Axillary buds and 2-mm shoot tips were directly immersed into the solutions, while small shoot tips(0.8 mm) were wrapped in a small strip of tissue paper.

After the loading treatment, the explants were exposed to PVS2 [30% (w/v) glycerol, 15% (w/v) EG, 15% (w/v) DMSO and 0.4 *M* sucrose; Sakai *et al.* 1990] for 10–60 min at 25°C. The explants were then suspended into 0.7 ml cryotubes with 0.5 ml fresh PVS2 and the tubes directly immersed into LN and kept for at least 1 h. After rapid warming in a water bath at 40°C, PVS2 was replaced with 5 ml of 1.2 *M* sucrose solution and kept for 10 min at 25°C.

Plant recovery and viability

Cryopreserved shoot tips were transferred onto two layers of sterilized filter paper disc over solidified MS supplemented with 0.3 *M* sucrose and 0.2% gellan gum at 25°C. After 2 days, the explants were transferred onto MS containing 0.1 *M* sucrose and 0.2% gellan gum. Cultures were kept in dim light for 10 days prior to exposure under the light conditions described above.

Shoot tips that showed normal shoot growth after 20 days of incubation were considered to have survived.

Results and discussion

Sensitivity of different sizes of shoot tips to PVS2

Vitrification solutions are potentially harmful due to the phytotoxic effects of individual components or their combined osmotic effects on cell viability (Towill and Jarret 1992; Matsumoto *et al.* 1994). Different explants, i.e. axillary buds and 2 mm-long and 0.8 mm-long shoot tips, were exposed to PVS2 for 10–60 min at 25°C to evaluate their sensitivity to PVS2. In all three of these explants shoot formation was 100% up to 20 min of exposure to PVS2, and the explants displayed a gradual decrease in survival after 30 min. The 0.8-mm shoot tips were most sensitive, followed by the 2-mm shoot tips, while the axillary buds were relatively tolerant. The survival rates of the materials after dehydration with PVS2 for 60 min were 65%, 80% and 95%, for the 0.8 mm- and 2 mm-shoot tips and axillary buds, respectively (Fig. 1).

Three different types of explants of taro which were exposed to PVS2 at 25°C for up to 20 min were able to resume 100% shoot growth. However, shoot tips of some plant species such as sweet potato and banana have been reported to be very sensitive to PVS2 treatment. Even treatment for 5 min at 25°C killed the shoot tips or reduced their survival rate significantly (Towill 1992; Panis 1995). Taro

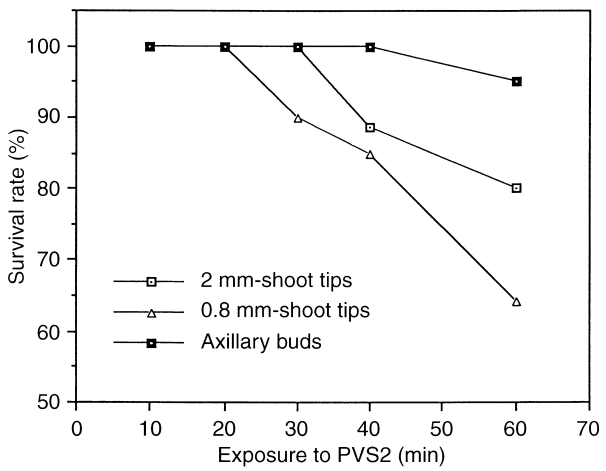

Fig. 1 Sensitivity of axillary buds and shoot tips to PVS2. Ten explants were tested for each treatment, and each data point represents mean of two replicates

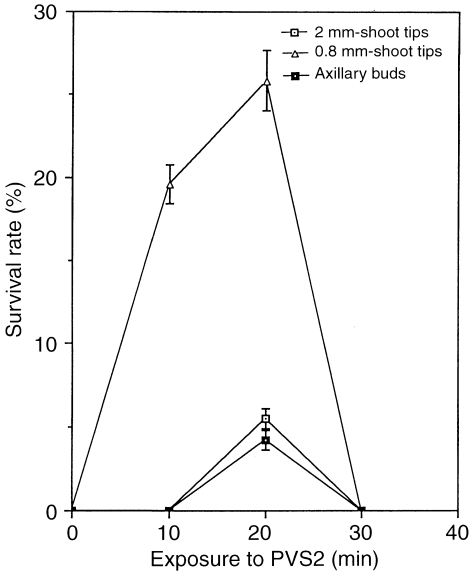

Fig. 2 Effects of the duration of PVS2 exposure on the survival of axillary buds and shoot tips cooled to –196°C. Explants were treated with PVS2 at 25°C for different periods of time before immersion into LN. Ten explants were tested for each of three replications. *Bar* represents standard error

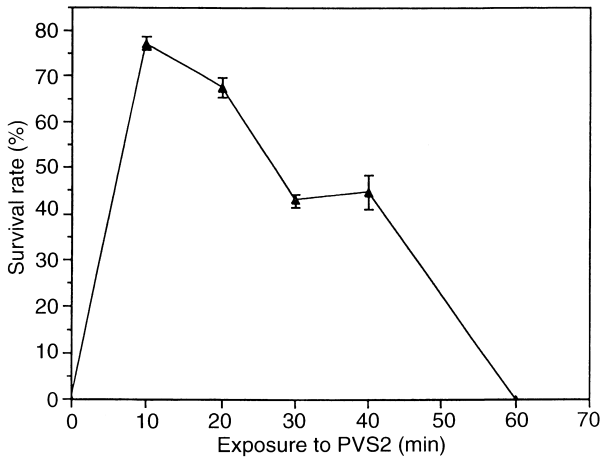

Fig. 3 Effects of the duration of PVS2 exposure on the survival of shoot tips, loaded and cooled to –196°C. Shoot tips were loaded with a mixture of 1.5 *M* glycerol, 0.4 *M* sucrose and 5% DMSO for 20 min and dehydrated with PVS2 for different periods of time at 25°C before immersion into LN. Ten shoot tips were tested for each of three replications. *Bar* represents standard error

shoot tips appear to be relatively insensitive to PVS2 compared to the shoot tips of sweet potato and banana.

Effects of different exposure time to PVS2

Survival rates of axillary buds and shoot tips cooled to –196°C after dehydration with PVS2 for different periods of time are shown in Fig. 2. Exposure to PVS2 for 20 min gave the highest survival rate for all of the explants tested, with the highest rate of survival, 25.8%, being obtained by 0.8 mm-shoot tips. Axillary buds and 2 mm-shoot tips had a very low survival rate of around 5%. Two to three weeks after reculturing, the surviving 0.8 mm-shoot tips reproduced small intact plantlets without intermediate callus formation. However, the surface of most of the surviving axillary buds and 2 mm-shoot tips turned dark brown, and these structures did not form new shoots but only green leaf-like tissues.

The insensitivity of taro shoot tips to PVS2 was considered to be due to the morphological characteristics of the plant. The thick and tubular petioles of young leaves with a waxy smooth surface that wrap tightly around the apical dome of taro may prevent permeation of the PVS2. For this reason, the axillary buds and the 2 mm-shoot tips of taro showed very low survival rates after cooling to –196°C. Most of the axillary buds and 2 mm-shoot tips which survived failed to reproduce new shoots but formed green leaf-like tissues, suggesting that only the outer tissues were fully vitrified. Thus, in the present study, the 0.8 mm-shoot tips, dissected out 0.5–0.8 mm in length with 1 to 2 leaf primordia, were selected as the proper experimental materials.

Table 1 Effects of loading solutions on the survival of vitrified taro shoot tips (cv. 'Eguimo') cooled to –196°C (*Gly* glycerol, *Suc* sucrose, *DMSO* dimethylsulfoxide)

Loading solutions[a]	Survival rate (%[b]±SE)
Untreated	23.3±1.5
L1: 1 *M* Gly+0.4 *M* Suc	69.5±2.0
L2: 1.5 *M* Gly+0.4 *M* Suc+5% DMSO	63.3±1.3
L3: PVS2 20%	40.0±0.0
L4: PVS2 20-40-60-80%	32.5±1.4

[a] Shoot tips were loaded with L1, L2, L3 for 20 min at 25°C followed by dehydration with PVS2 before immersion into LN. For L4, each solution level was applied for 5 min at 25°C
[b] Ten meristems were tested for each of three replications

Effects of loading treatment

Post-LN survival rate by vitrification was increased by cryoprotecting (loading) the shoot tips with a mixture of 1.5 *M* glycerol, 0.4 *M* sucrose and 0.5% DMSO for 20 min at 25°C before being treated with PVS2 for different periods of time. As shown in Fig. 3, the loading treatment was very effective in enhancing the survival of shoot tips in comparison with the unloaded shoot tips (0.8 mm-shoot tips) in Fig. 2. The highest survival rate, 77%, was obtained for loaded shoot tips treated with PVS2 for 10 min at 25°C.

To optimize the factors controlling the survival of shoot tips cooled to –196°C by vitrification, we compared the cryoprotective effects of four different loading solutions. As shown in Table 1, the highest survival rate, 69.5%, was observed in shoot tips treated with the L_1 solution, followed by the L_2 solution (63.3%). Based on these results, a mixture of 2 *M* glycerol plus 0.4 *M* sucrose (L_1) was

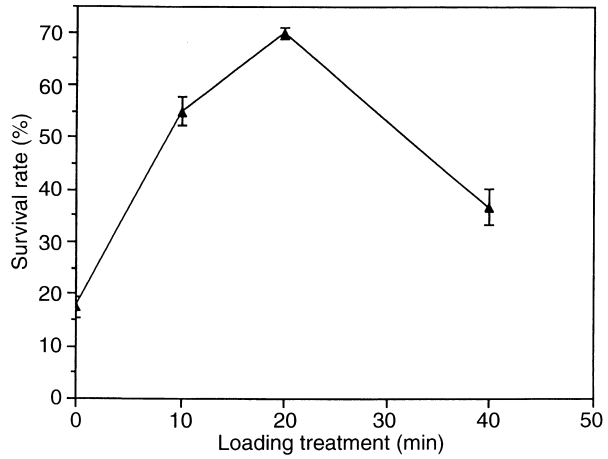

Fig. 4 Effects of the duration of loading treatment on the survival of shoot tips cooled to –196°C. Shoot tips were loaded with a mixture of 2 *M* glycerol and 0.4 *M* sucrose for different periods of time and dehydrated with PVS2 for 10 min at 25°C before immersion into LN. Ten shoot tips were tested for each of three replications. *Bar* represents standard error

adopted as the loading solution for taro shoot tips in the following experiments.

The optimum time for loading treatment was determined by treating shoot tips with a mixture of 2 *M* glycerol and 0.4 *M* sucrose at 25°C for different periods of time before being treated with PVS2. The shoot tips treated for 20 min showed the highest survival rate (Fig. 4).

For successful cryopreservation by vitrification of plant tissues, it is essential to carefully control the dehydration procedures so as to provide enough dehydration while at the same time preventing injury caused by chemical toxicity and sudden osmotic stress. Recently, shoot tips of wasabi (Matsumoto *et al.* 1994) and lily (Matsumoto *et al.* 1995a) have been effectively cryopreserved by the addition of a loading treatment before the exposure to PVS2. In the present study, the loading treatments, especially those with a mixture of 2 *M* glycerol plus 0.4 *M* sucrose, were essential to obtain a high survival rate in cryopreservation by vitrification of taro shoot tips.

Table 2 Effects of age of donor plants on the survival of vitrified taro shoot tips (cv 'Eguimo') cooled to –196°C

Age of donor plants	Survival rate[a] (%[b]±SE)
1 week old	46.6±2.5
1 month old	45.0±2.8
2 months old	63.3±1.4
3 months old	75.0±2.0

[a] Shoot tips were loaded with 2 *M* glycerol+0.4 *M* sucrose for 20 min followed by PVS2 treatment for 10 min at 25°C before immersion into LN
[b] Ten meristems were tested for each of three replications

Table 3 Effects of preculture with sucrose on the survival of vitrified taro shoot tips (cv 'Eguimo') cooled to –196°C

Sucrose concentration (*M*)	Survival rate[a] (%[b]±SE)
None	66.6±2.3
0.3 *M*	83.1±2.3
0.5 *M*	13.3±0.9
0.7 *M*	0.0±0.0

[a] Precultured shoot tips with different concentrations of sucrose applied for 1 night 16 h at 25°C were loaded with 2.0 *M* glycerol+0.4 *M* Sucrose for 20 min and dehydrated with PVS2 for 10 min at 25°C before immersion into LN
[b] Ten meristems were tested for each of three replications

Table 4 Survival of cryopreserved shoot tips from different genotypes of *Colocasia esculeta var antiquorum*

Cultivar names	Scientific names	Survival rate[a] (%[b]±SE)
Eguimo	*C. esculenta* var *antiquorum*	77.2±1.5
Dodare	*C. esculenta* var *antiquorum*	66.6±4.3

[a] Shoot tips were (1) precultured on 0.3 *M* sucrose overnight, (2) loaded with 2 *M* glycerol+0.4 *M* sucrose for 20 min at 25°C, (3) dehydrated with PVS2 for 10 min at 25°C before immersion into LN
[b] Ten shoot tips were tested for each of four replications

Effects of age of shoot-tip donors

Effects of age of shoot-tip donor plants on the survival of shoot tips cooled to –196°C was investigated. The survival rate increased gradually as the age of the donor of the shoot tip increased – from 46% (1-week-old plants) to 75% (3-month-old plants) (Table 2).

The higher survival rates obtained in the older shoot-tip donor plants suggested that physiological conditions are very important factors in increasing the tolerance to the cryogenic procedures. We observed that the 2-month- and 3-month-old *in vitro* plants had initiated tuberization of the main corm and that carbohydrate accumulation in the cells and organs at this stage of growth could be very active. It was also observed that the shoot tips from younger plants tended to be smaller and more watery and translucent in comparison with the ones from older plants. Further studies on suitable physiological status and proper conditioning of shoot-tip donor plants could be a key point to raise tolerance to LN treatment in the cryopreservation of tropical and subtropical species.

Effects of preculture with sucrose

Precultures of excised shoot tips with different concentrations of sucrose prior to loading treatment have been reported to be effective in improving post-LN survival rates

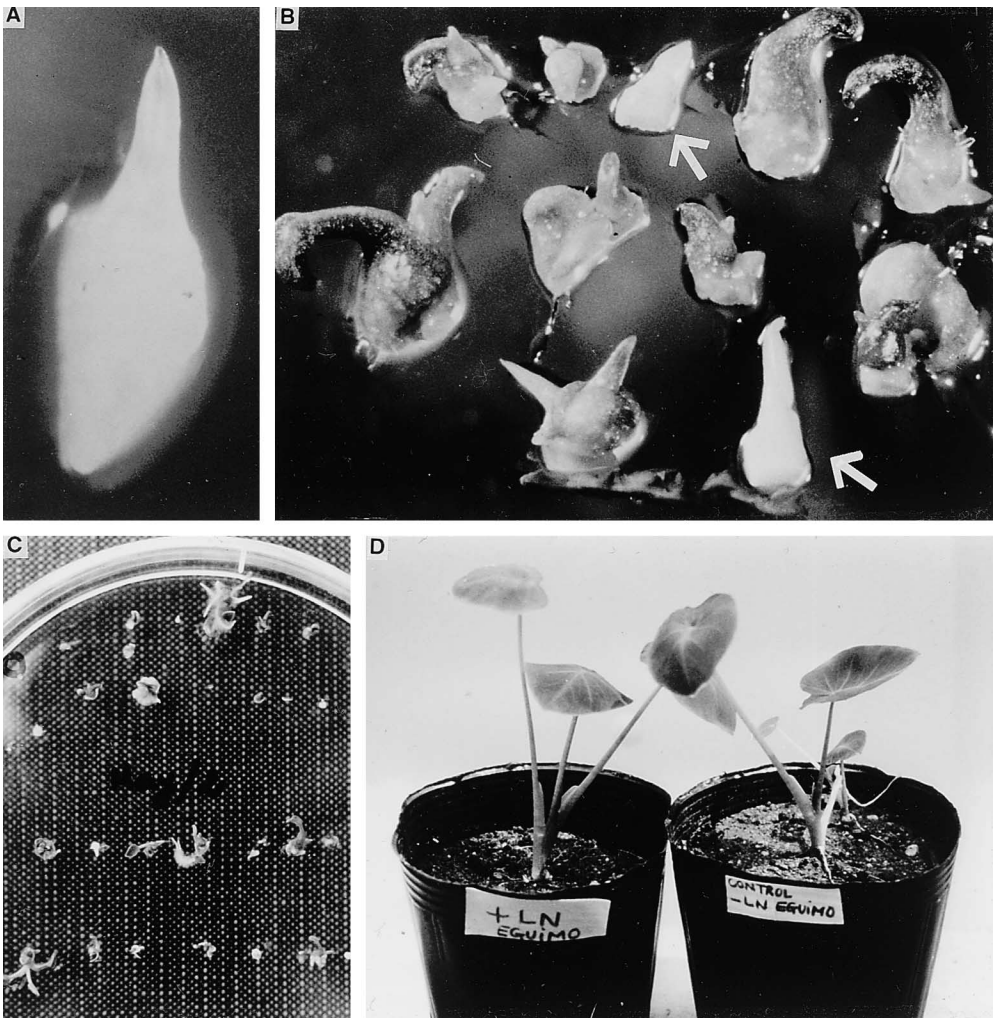

Fig. 5 **A** Dissected taro (*C. esculenta* var *antiquorum* cv 'Eguimo') shoot tips (ca. 0.8 mm in length) suitable for cryopreservation by vitrification. **B** Shoots formed from cryopreserved shoot tips by vitrification, at 7 days after culture. Dead shoot tips turned white (indicated by *white arrows*), whereas those which survived showed a green color. **C** General view of plantlets recovered from cryopreserved shoot tips of taro by vitrification at 15 days after culture. Rooting was already initiated at this stage. **D** *Left* Taro plants developed from cryopreserved shoot tips by vitrification, *right* untreated control

of some species (Matsumoto *et al.* 1994, 1995a; Yamada *et al.* 1991). For taro shoot tips, preculturing with 0.3 *M* sucrose for 16 h considerably improved the survival rate, which reached a value of 83%, while preculture with 0.5 *M* and 0.7 *M* sucrose decreased the survival rate (Table 3).

Application of the protocol to a different genotype

This cryogenic protocol was effectively applied to another genotype of *C. esculenta* var *antiquorum*, cv 'Dodare', and a survival rate of 67% was recorded (Table 4).

The vitrification protocol developed here appears to be promising as a practical method for *C. esculenta* (L.) Schott. A polymorphic species, *C. esculenta* contains two botanical varieties, i.e. var *esculenta* and *antiquorum* (Purseglove 1972). The two cultivars used in this study belong to *C. esculenta* var *antiquorum*, a triploid taro grown commonly in China, Japan and the West Indies, which produces a relatively small central corm and many lateral cormels. The application of the identified protocol to var *esculenta*, a diploid taro popular in tropical areas, and other species belonging to *Aracea*, such as *Alocasia*, *Cytosperma* and *Xanthosoma*, is in progress.

Optimized protocol and regrowth of plants

On the basis of the above investigations, the factors controlling the survival of shoot tips cooled to –196°C by vitrification were optimized as follows: (1) use of shoot tips with 1–2 leaf primordia from 3-month-old plants (Fig. 5A); (2) preculture with 0.3 *M* sucrose for 16 h; (3) loading treatment with a mixture of 2 *M* glycerol and 0.4 *M* sucrose for 20 min at 25°C; (4) dehydration with PVS2 for 20 min at 25°C prior to immersion into LN. Successfully vitrified and warmed shoot tips had a green color continuously after plating (Fig. 5B), resumed shoot-growth within about 7 days and produced roots within 2 to 3 weeks without intermediate callus formation. Almost all the shoot tips formed roots on hormone-free solidified MS (Fig. 5C) and were successfully transferred to soil in pots. No morphological abnormalities were observed in the plants developed from cryopreserved shoot tips (Fig. 5D).

The survival rate obtained under our optimal cryogenic conditions are reasonably high for cryopreserved of shoot tips comparing with results obtained with many other species. However, the protocol still gave replication-to-replication variation, i.e. 66%–100% (avg. 79.5%), in survival after LN exposure although we carried out the experiments with the most possible care to make it constant. Similar variations were pointed out by Towill (1990, 1992) for mint and sweet potato. Limited number of samples per each experiment is, certainly, one of the reasons, but we believe that it may be essential to use shoot-tip donor plants of an optimal physiological status for cryostorage. For example, cold hardening for cold hardy species has been reported as essential not only to increase the survival rate after LN exposure but also to stabilize the survivals (Matsumoto *et al.* 1995a, Niino *et al.* 1992). There is a need for further studies on clarifying the optimal physiological conditions for cryopreservation, where dessication tolerance should be highly expressed, especially for less hardy plants, tropical and subtropical species.

We demonstrated here that the vitrification procedure for cryopreservation, which had been successfully applied to different species of temperate crops, could also be used for the cryopreservation of taro, an established tropical crop. It was also confirmed that cryopreserved taro shoot tips by vitrification usually produced shoots directly without intermediate callus formation. Thus, vitrification is considered to be a potentially valuable cryogenic method for the cryopreservation of meristems. Further studies are needed to expand the applicability of this procedure to other vegetatively propagated tropical species.

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