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STUDIES ON FREEZE-PRESERVATION OF FRUIT TREE GERMPLASM

III Freeze-preservation of grape shoot tips

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Introduction

Maintenance of varieties of vegetatively-propagated perennial horticultural crops through a conventional method of cultivation requires large amounts of energy, area and cost. Reriable and routine methods are required to avoid the inconveniences and the damages due to unusual climate, disease and insects. In recent years, plant germplasm freeze-preservation at superlow temperatures has been studied as one of such methods[1-10,12]. However, no papers are available on the survival of grape shoot tips frozen in liquid nitrogen (LN). Therefore, in this study, we have attempted to examine the survival of prefrozen or LN-frozen grape shoot tips which were excised from autumn or winter field-grown grape vines, and also to test long term preservation in LN.

Materials and Methods

One-year-old vines were obtained from nine-year-old grape plants (*Vitis labrusca* L.) growing in the Experimental Orchard of Hokkaido University (Yoichi-cho, Hokkaido, Japan). 'Campbell Early' and 'Buffalo' were taken on 18 Sept., and 'Delaware', 'Buffalo' and 'Campbell Early' were used on 12 Nov. and on 13 Dec. Materials for long term storage were collected on 20 Mar. from 'Buffalo'.

Small vine segments (3 cm in length) with a node were excised from the vines, and the bark and 2-3 leaflets of the segments were removed. Then the segments were sterilized with 70% ethanol for 30 sec, and subsequently with 10% sodium hypochlorite solution (1% available Cl, with 0.1% Tween 20) for 15 min. The shoot tips 1 to 2 mm in length were prepared aseptically and immersed in about 1-ml freezing solution (containing 10% DMSO and 60 g/l glucose as cryo-protectant) in a straw (made of plastic, 123 mm in length, 4 mm in diameter) for 2 hrs at room temperature. After that, the samples were cooled down to the prescribed temperatures with a programing freezer (prefreezing, cooling rate: 0.5°C/min). LN-immersing treatment (cooling rate: 500°C/min) was carried out

by plunging the straws into LN after the prefreezing. The frozen samples were thawed rapidly in water at 38°C (rewarming rate: 420°C/min), transferred onto solid medium (at pH 5.7) containing MS medium, 2 mg/l N^6-benzyladenine, 30 g/l sucrose and 7 g/l agar, and cultured at 25°C under 16-hr daylength (4,000 lx, fluorescent lamp).

Results

In the shoot tips obtained on 18 Sept., about half of 'Buffalo' and most of 'Campbell Early' frozen to −30°C lost their viability (Fig. 1). Furthermore, the growth rates of survived shoot tips of both cultivars frozen to −20° and −30°C was very slow, and finally they stopped growing after minimal leafing.

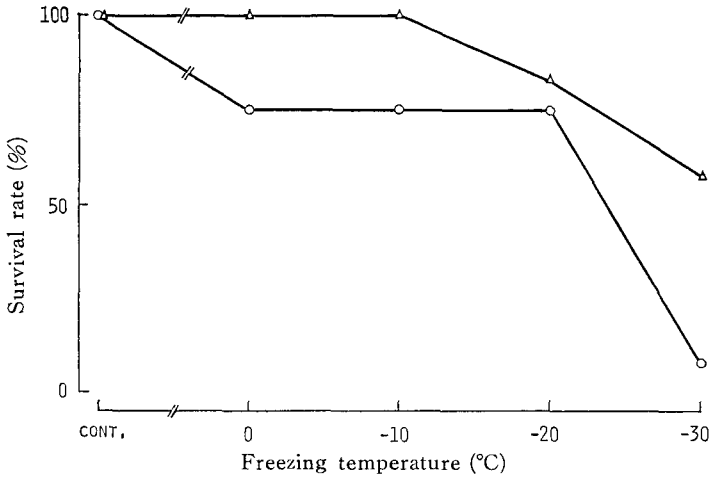

Fig. 1. Effect of freezing temperatures on survival of grape shoot tips collected on 18 Sept. Control stands for a shoot tip cultured without freezing. Cultivars are shown as follows: ○ stands for 'Campbell Early'; △, 'Buffalo'.

The survival rates of the three cultivars ('Delaware', 'Buffalo' and 'Campbell Early') obtained on 12 Nov. were 100% at −20°C, and more than 75% at −30° and −40°C, not followed by LN immersion (Fig. 2). Many shoot tips survived after LN immersion. It was observed especially in 'Delaware' and 'Buffalo' that prefreezing temperatures were more effective in the growth process than in survival rate. Shoot tips prefrozen to −30°C regrew rapidly to develop normal shoots, while those prefrozen to −40°C survived for nearly a month to bear two to three leaflets and then cease to grow. In 'Campbell Early', most of frozen shoot tips, except for those of control, showed callus formation.

The shoot tips collected on 13 Dec. also indicated high survival rates (Fig. 2). In the case of being frozen in LN finally the 'Delaware' shoot tips prefrozen to −20°, −30° and −40°C showed the same survival rates and growth processes.

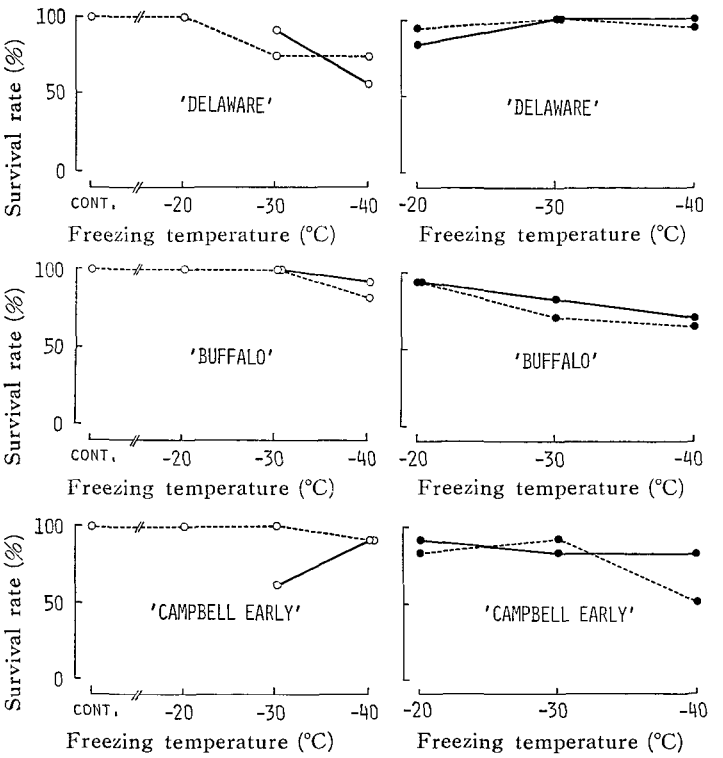

Fig. 2. Effect of prefreezing temperatures and LN-immersion on survival of grape shoot tips. Survival rates at various freezing temperatures are shown as follows: dotted lines, prefreezing; solid lines, LN-immersion following prefreezing. Collecting times of plant materials are shown as follows: ○ stands for 12 Nov.; ●, 13 Dec.

However, the 'Buffalo' shoot tips which were prefrozen to −20°C before LN immersion grew slowly, compared with the shoot tips prefrozen to −30°C or −40°C, and finally stopped growing after minimal leafing. Most shoot tips of 'Campbell Early' also formed calluses as in the previous experiment on 12 Nov.

The effect of long term storage in LN on the survival rates was tested (Table 1). After 18-month storage in LN,

TABLE 1. Effect of liquid nitrogen-immersion periods on survival of grape shoot tips[z] frozen at −196°C[y]

Cultivar	Survival rate (%)			
	Periods of freezing in LN			
	one day	6 months	12 months	18 months
'Buffalo'	86.7 (13/15)[x]	80.0 (12/15)	78.6 (11/14)	80.0 (12/15)

[z] Collected on 20 Mar.
[y] Prefrozen to −30°C.
[x] Parenthesis shows shoot tips survived/shoot tips used.

the survival rate remained considerably high. The survival rates after 6, 12 and 18 months of storage were approximately 80%.

Discussion

In the autumn shoot tips, a large part of meristems was injured by freezing, because freezing resistance of the tips were not sufficient to tolerate freezing. Therefore, the shoot tips made their leaf primodia elongate, but showed no shoot formation. Regarding the effect of temperature of prefreezing on survivals of the November or December-collected shoot tips especially in 'Buffalo', it is assumed that intracellular freezing was induced in the cells of shoot tips prefrozen to −20°C when plunged into LN, because the cells might not be dehydrated sufficiently by the prefreezing[11]. It is probably due to low freezing resistance that callus induction and leaf primodia elongation without shoot formation are observed in 'Campbell Early' shoot tips frozen down to −20°, −30°, −40° and −196°C.

Winter dormant buds (meristems) have often been used as plant materials in studies of freeze-preservation of woody plants, because they have high freezing resistance[5,7,10]. However, the correlation between hardening condition and physiological-structural mechanisms of freezing resistance are not yet explained clearly. In order to clarify this phenomenon, the authors are investigating the seasonal changes in endogenous substances of shoot tips and the condition to harden *in vitro*-cultured plants, and will simultaneously continue to carry out further experiments on long term storage.

Summary

For the purpose of establishing freeze-preservation method of grape shoot tips in liquid nitrogen (LN), we investigated survival of artificially frozen-thawed shoot tips derived from grape vines growing in the field in seasonally different periods (Sept., Nov. and Dec.), and also examined long term storage in LN.

Shoot tips (1 to 2 mm) were excised from vines of nine-year-old grape plants (*Vitis labrusca* L.; 'Buffalo' and 'Campbell Early' in Sept., and 'Delaware', 'Buffalo' and 'Campbell Early' in Nov. and Dec.), immersed in freezing solution containing 10% DMSO and 60 g/l glucose as cryoprotectant at 20°C for 2 hrs, then cooled down to prescribed temperatures (prefreezing, cooling rate: 0.5°C/min). LN-immersing treatment was carried out by plunging the samples into LN (−196°C) after prefreezing (cooling rate: 500°C/min). The frozen samples were thawed in water at 38°C (rewarming rate: 420°C/min) before culture on solid medium.

In Sept., about half of 'Buffalo' shoot tips and most of 'Campbell Early' shoot tips lost their viability at −30°C (without LN immersion). In contrast, in Nov. and Dec., many shoot tips survived even after LN immersion. The optimum prefreezing temperature before LN immersion was −30°C in 'Delaware'

and 'Buffalo'. Almost all 'Campbell Early' shoot tips frozen to any temperature in this experiment formed calluses.

The survival rate of 'Buffalo' shoot tips was 80% after 18-month long term storage in LN.

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