Survival and Shoot Formation in Vitro of Pear Winter Buds Cryopreserved in Liquid Nitrogen

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Additional index words. *Pyrus serotina*, shoot-tip culture, vegetative winter bud

Abstract. Vegetative buds of pear (*Pyrus serotina* Rehder, cv. Senryo) associated with shoot tissue were frozen stepwise to -40°C , immersed in liquid N₂, and thawed either at 37°C in water or at 0°C in air. Survival and shoot formation on thawed material was higher when preliminary cooling lasted 12 to 48 h rather than 0.5 to 2 h. The proper thawing temperature depended on the degree of cooling before cryopreservation. A few whole plants were recovered from the cryopreserved buds.

Fruit tree germplasm is being conserved mainly in the form of a field genebank, which requires much labor, money, and land. In addition, this germplasm is apt to be lost by attack from pathogens, pests, and environmental disasters. The advisory committee of the International Board for Plant Genetic Resources (1986) urged the development of an alternative, i.e., an in vitro genebank. Although storage of shoots in vitro under slow growth conditions has been reported in several fruit tree species (Banerjee and Langhe, 1985; Barlass and Skene, 1983; Marino et al., 1985; Monette, 1986), there have been relatively few reports on cryopreservation (Katano et al., 1983; Reed, 1990; Reed and Lagerstedt, 1987; Sakai and Nishiyama, 1978). Yakuwa and Oka (1988) recently reported that mulberry winter buds associated with shoot tissue were capable of regenerating plants after storage in liquid nitrogen (LN₂). In this paper, cryopreservation of pear winter buds is reported.

Shoots of 'Senryo' pear were harvested in February, when buds were dormant, in an orchard in Yōichi-machi, Hokkaido, Japan, and immediately sent to our laboratory. Shoot segments (1.0-1.5 cm long) bearing a vegetative winter bud were put in canisters and subjected to preliminary stepwise freezing down to -40°C before storage in LN₂. The preliminary freezing was accomplished by transferring the canisters through a series of electrically controlled freezing chambers providing 0 ± 1 , -5 ± 1 , -10 ± 1 , -20 ± 1.5 , -30 ± 1.5 , and $-40 \pm 1.5^{\circ}\text{C}$. The canisters were kept at each temperature for 1 day, followed by immersion in LN₂ for at least 1 day. Then the shoot segments were

warmed either slowly at 0°C in air for more than 3 h or rapidly in agitated water at 37°C for 5 min. The thawed segments were surface-sterilized with 70% ethanol for 1 min and sodium hypochlorite solution (0.5% effective chlorine concentration) for 30 min, and rinsed three times with sterile distilled water. Shoot tips ≈ 1 mm in diameter were excised and placed on culture media. Five milliliters of Murashige and Skoog (MS) (1962) or woody plant medium (WPM) (Lloyd and McCown, 1980), each containing BA at $1 \text{ mg} \cdot \text{liter}^{-1}$ and 2.5% sucrose, was used for shoot-tip culture. Minor organic addenda were (in $\text{mg} \cdot \text{liter}^{-1}$) 1 thiamine-HCl and 100 inositol. The media were solidified with 0.7% agar (Wako Chemical Co., Osaka, Japan). The pH was adjusted to 5.6 (MS) or 5.2 (WPM) before autoclaving at 120°C for 15 min. The cultures were maintained at 25°C under a 16-h photoperiod ($52 \mu\text{mol} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) using warm-white fluorescent tubes. After 8 to 10 weeks of culture, the number of shoot tips surviving, developing leaves, and forming shoots was recorded. The specimens that remained green during the entire culture period were regarded as surviving, regardless of subsequent growth into leaves or shoots.

Specimens that had discernible shoot axes were regarded as having formed shoots and were distinguished from those just developing leaves, which remained in rosettes.

When the regenerated shoots were 1.5 cm long, after 10 to 12 weeks in culture, they were transferred to rooting medium composed of modified MS (half-strength major inorganic salts) containing 1 mg α -naphthaleneacetic acid (NAA)/liter. Rooted plants were planted in Jiffy pots filled with 1 vermiculite : 1 peat moss (v/v) and acclimatized in plastic containers that were placed in a humidified atmosphere and covered with polyethylene film that was opened occasionally. Acclimatized plants were finally planted in pots and grown outdoors.

Initial growth was slightly delayed in the frozen shoot tips. Light-yellow, young leaves that surrounded the shoot apical dome turned green within 2 weeks in culture and continued to enlarge, but the bud remained in a rosette for 4 weeks, or sometimes much longer, until shoot elongation took place, usually after 4 to 6 weeks of culture. Few of the nonfrozen (control) leaf rosettes produced shoots. For example, as few as 20% of the nonfrozen specimens produced shoots (Table 1). A similar recalcitrance to form elongated shoots in the shoot-tip culture of *Pyrus serotina* winter buds was reported by Yotsuya et al. (1984).

Preliminary freezing temperatures, at which the buds were transferred to LN₂, and thawing temperatures affected survival, leaf development, and shoot formation (Table 1). The buds frozen to -10°C never survived, regardless of the thawing temperatures. When they were frozen to -20°C , the thawing temperature was quite critical; only rapid thawing at 37°C gave sufficient viability leading to shoot formation. In contrast, when freezing to -30 or -40°C , slow thawing at 0°C was superior to rapid thawing for achieving a higher rate of survival and leaf development. The number of buds forming shoots was considerably less than the number of surviving or developing leaves, because many specimens remained in the rosette state. WPM

Table 1. Effects of preliminary freezing temperature before holding in LN₂ and thawing temperature on growth of winter buds of 'Senryo' pear.

		MS medium			WP medium		
Temp ($^{\circ}\text{C}$) ^a		Survival (%)	Leaf development (%)	Shoot formation (%)	Survival (%)	Leaf development (%)	Shoot formation (%)
Preliminary	Thawing						
-10	0	0	0	0	0	0	0
	37	20	0	0	20	0	0
-20	0	50	30	0	60	50	0
	37	90	80	20	80	80	50
-30	0	80	60	20	90	60	40
	37	80	30	20	80	60	10
-40	0	60	60	10	80	70	30
	37	30	30	10	20	20	10
Nonfrozen	---	84	43	22	72	38	22
Significance ^b		**	**	NS	**	**	*

Received for publication 19 Mar. 1990. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

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^aNumber of samples for preliminary freezing treatments and nonfrozen control were 10 and 37, respectively.

^bChi-square test.

NS, **, ***Nonsignificant or significant at $P = 0.05$ or 0.01 , respectively.

Table 2. Effect of rapidity of cooling on post-thawing growth of cryopreserved winter buds of 'Senryo' pear.¹

Cooling duration (h)	No. samples	Survival		Shoot formation	
		No.	Percent	No.	Percent
0.5	60	0	0	0	0
2	59	9	15	0	0
12	50	31	62	4	8
24	58	35	60	3	5
48	60	40	67	2	3
Significance ²		**		*	

¹The specimens were frozen stepwise (from 0.5 to 48 h at each of 0, -5, -10, -20, and -30C before being frozen in LN₂ for 1 day). After being thawed slowly at 0C in air, the shoot tips were cultured on WPM for 8 weeks. Numbers are the sum of three experiments.

²Chi-square test.

³Significant at $P = 0.05$ or 0.01 , respectively.

gave somewhat better results than MS medium.

The intervals during which the specimens were kept at each temperature, from -5 to -30C, was varied between 0.5 and 48 h; all were thawed slowly at 0C after immersion in LN₂. When the specimens were cooled for 2 h or less before immersion in LN₂, the survival rate was less than when cooling lasted 12 h or longer; then, > 60% of the specimens survived and some of them formed shoots (Table 2). Excised buds associated with shoot tissues presumably take longer to dehydrate extracellularly than small shoot tips. Therefore, the specimens used in this experiment needed slower cooling for survival than the excised small shoot tips conventionally used for cryopreservation (Katano et al., 1983; Reed, 1990).

After 8 to 10 weeks of culture or longer, a total of 50 shoots derived from various experiments were transferred to a rooting medium. Rooting occurred in 13 shoots (26% rooting efficiency) 10 to 14 days after transfer, and two plants were successfully potted out in soil.

Since the frequency of shoot formation in our study was low, even for nonfrozen buds, developing a more effective culture medium, one that will facilitate normal shoot growth of winter buds of *Pyrus*, is necessary. Nevertheless, the method described here, in which naturally cold-hardened winter buds were used for preservation, has advantages. The preparation of specimens is simple and they can be handled easily without using cryoprotectants and expensive programmable freezing units, either or both of which are almost exclusively used in conventional cryopreservation of meristematic tissue (Reed, 1990).

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