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# Long Term Storage of Pear (Pyrus spp.) Shoot Cultures In Vitro by Minimal Growth Method

**Seibi OKA*[1] and Takao NIINO*[2]**

*[1] Department of Biotechnology, National Institute of Agrobiological Resources (Tsukuba, Ibaraki, 305 Japan)

*[2] Department of Crop Breeding, Tohoku National Agricultural Experiment Station (Shinjo, Yamagata, 996 Japan)

**Abstract**

Pear (*Pyrus serotina* cv. Senryo) shoots grown on MS medium containing 2.5% sucrose, 1 mg/l BA and solidified with 0.8% agar (control medium) were stored at 5 different temperatures for 64 weeks. The shoots did not survive at 0? for more than 4 weeks. At intermediate temperatures such as 18°C and 10°C, the survival rates of the shoots gradually declined after 16 and 32 weeks, respectively. Both survival and shoot regeneration rates were the highest in the shoots stored at 5°C under an 8 h light and 16 h dark regime. Modified media containing Paclobutrazol (PP333), Uniconazole-P (S-327D), 10% sucrose, 2% agar or 1/4 strength MS salts were more effective for 2-year storage compared with the control medium. Pyrus communis (cv. Winter Nelis) shoots stored at 5°C for 64 weeks also regenerated shoots normally. The established storage method was successfully applied to nearly 200 pear accessions conserved in the field genebank.

**Discipline:** Biotechnology/Genetic resources
**Additional key words:** growth retardant, low temperature, shoot regeneration, sucrose concentration

## Introduction

The germplasm of vegetatively propagated plants including fruit trees is basically conserved in orchards as a field gene bank[4]. This system, however, requires a large land area and a great deal of labor for growing plants safely. *In vitro* slow growth storage of plant germplasm has been developed for several fruit trees[3,5,11,12]. Although cryopreservation methods have been reported in pear[7,9,10], short to medium term storage of pear germplasm can be more readily achieved by applying the slow growth method and is useful for rapid distribution as a working collection. The simplest methods to slow down shoot growth *in vitro* are the reduction of temperature and culture on media under sub-optimal nutritional and physical conditions.

In the present paper, the effects of different temperatures, light conditions, and some modifications of the medium components on the survival and regeneration of pear shoots are described. In addition, the slow growth method was actually applied to practical storage of over 200 pear accessions conserved in the field genebank.

## Materials and methods

*1) Initiation of shoot culture*

Branches were harvested in February (post-dormant stage) for *Pyrus serotina* (cv. Senryo) and in July (current growth stage) for *P. communis* (cv. Winter Nelis). Shoot meristems about 1 mm in diameter were excised from winter buds of Senryo and terminal buds of Winter Nelis that were sterilized with a sodium hypochlorite solution (1% effective chlorine) for 10 min and rinsed 3 times with sterilized water. They were cultured on solid (0.8%

Present address:
*[2] Department of Upland Farming, Tohoku National Agricultural Experiment Station (Fukushima, Fukushima, 960-21 Japan)

agar) Murashige and Skoog[8] (MS) medium containing 2.5% sucrose and 1 mg/l benzyl adenine (BA). The pH of the medium was adjusted to 5.8 before autoclaving at 120°C for 15 min. The cultures were maintained at 25°C under 16 h illumination with fluorescent light (3,000 lux). Shoots were proliferated by transferring the cultures to MS medium with 2.5 mg/l BA every month.

*2) Storage of shoots*

(1) Experiment 1

Shoots of Senryo, 5–10 mm long, were obtained from the proliferated shoot cultures and placed in a flask (100 ml) containing 30 ml of MS medium with 2.5% sucrose and 1 mg/l BA. The flasks covered with aluminium foil only were incubated at 25, 18, 10, 5 and 0°C. The humidity level was not controlled. The light conditions provided by warm-white fluorescent lamps of 2,000–4,000 lux were 16 h day/8 h night (25, 18, and 10°C), 8 h day/16 h night (5°C), and 24 h night (5 and 0°C). Survival rate was estimated based on the number of shoots with green terminal and/or lateral buds prior to transfer to shoot regeneration medium 16, 32, 48, and 64 weeks after incubation. Shoots of Winter Nelis were also stored at 5°C for 16, 32, 48, and 64 weeks, both under dark and under light conditions. Each treatment consisted of 3 replicates (flasks) with 5–8 shoots per flask.

(2) Experiment 2

Shoots of Senryo were stored under light conditions at 5°C for 2 years on various kinds of modified MS media to prolong the duration of storage: MS medium containing 2.5% sucrose and 1 mg/l BA (control medium) was either modified with 1/4 strength inorganic salts or without BA, or supplemented with 25 mg/l BA, 1 and 10 mg/l abscisic acid (ABA), 1 mg/l Paclobutrazol (PP333), 1 mg/l Uniconazole-P (S-327D) (Sumitomo Chemical Co., Ltd.), 2% agar, and 0 and 10% sucrose.

*3) Regeneration of shoots from stored shoot cultures*

After the storage period, the shoots were trimmed to a length of 8–15 mm by removing their basal portion if necessary, and cultured at 25°C for 4 weeks on a shoot regeneration medium containing the same components as the shoot proliferation medium.

*4) Practical use of slow growth method for germplasm storage*

*In vitro* shoot cultures were established for as many as 227 accessions of *Pyrus* spp. which had been maintained as a field genebank at Tohoku National Agricultural Experiment Station (Shinjo, Yamagata, Japan). Excised shoot tips of these accessions were stored at 5°C under light conditions (8 h photoperiod) for 6, 12, and 24 months, when the survival rate was estimated.

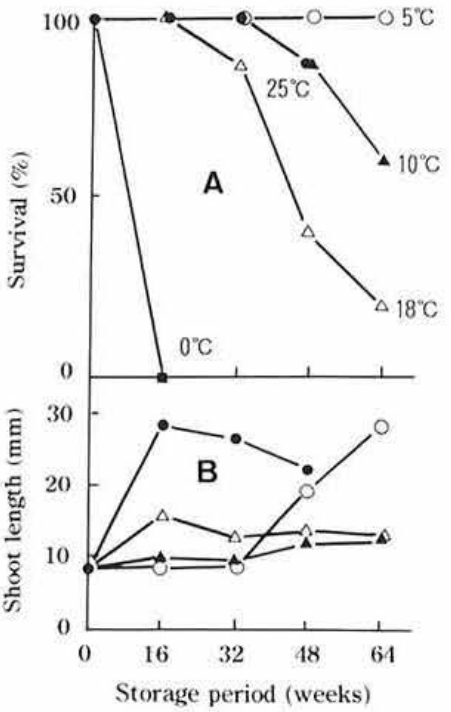


Fig. 1. Survival and elongation of shoots in *Pyrus serotina* (cv. Senryo) after storage at different temperatures for 16, 32, 48 and 64 weeks
All the shoots were stored under light conditions on MS medium containing 2.5% sucrose and 1 mg/l BA.
■ (0°C), ○ (5°C), ▲ (10°C), △ (18°C), ● (25°C).

## Results

*1) Experiment 1*

Periodic changes in the survival rate, shoot growth *in vitro*, and morphological appearances of the shoots stored at different temperatures are shown in Fig. 1 and Plate 1, respectively. Shoot tips of Senryo kept at 0°C became necrotic within 4 weeks. At 25°C many leaves in the lower part of the shoots showed necrosis, while 100% of the shoots retained green buds in the uppermost portion for up to 32 weeks and then a slight reduction in the survival rate was observed after 48 weeks of culture. At an intermediate temperature of 18°C, severe leaf

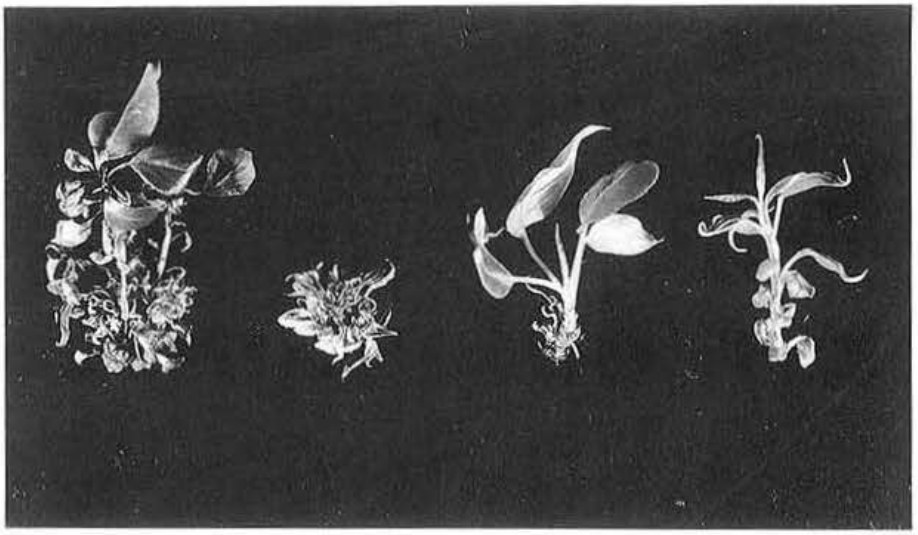

Plate 1.
Morphological appearance of shoots of Senryo stored at 25°C, 18°C, 10°C and 5°C (from left to right) for 48 weeks

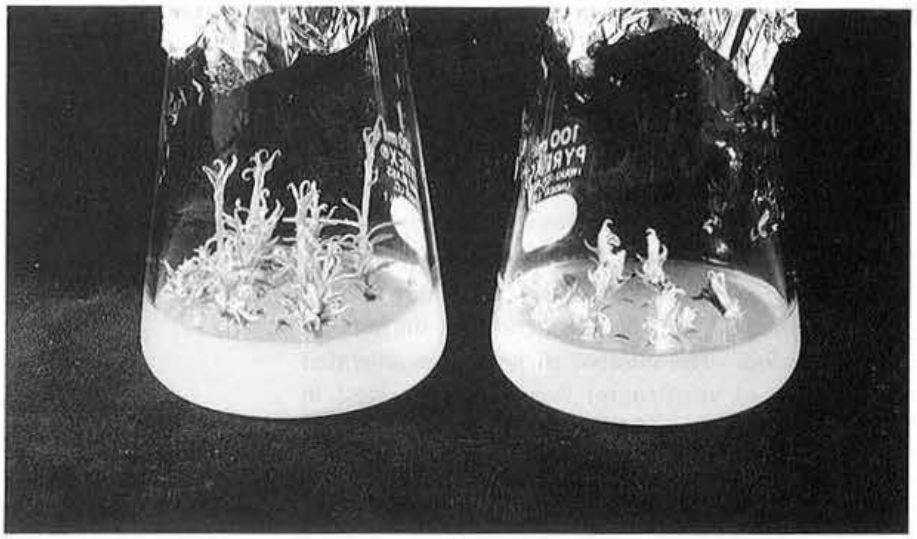

Plate 2.
Shoots of Senryo stored at 0°C for 64 weeks under light (left) and dark (right) conditions

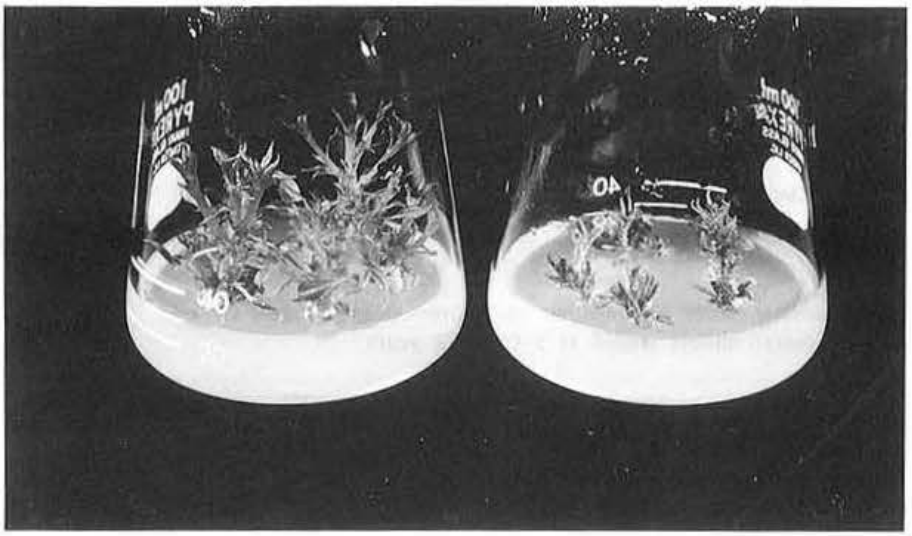

Plate 3.
Shoot regeneration of Senryo from shoots stored at 5°C for 64 weeks under light (left) and dark (right) conditions

senescence along the entire shoot axis periodically occurred, and the survival rate, which was rather lower than that at 25°C, decreased rapidly after 32 weeks of culture. The shoots incubated at 10°C showed defoliation of lower leaves associated with the induction of dormancy of terminal buds, which sometimes subsequently resumed growth, producing stunted internodes. The most suitable temperature and light conditions for long term storage were 5°C under light conditions, where 100% survival rate was obtained after 64 weeks of culture. However, the stored shoots began to elongate after 32 weeks onwards even at this low temperature and reached a length of about 30 mm after 64 weeks (Plate 2). When the shoots were stored under dark conditions at 5°C for 64 weeks, shoot elongation was minimal and the survival rate decreased from 32 weeks onwards compared with the shoots stored under light conditions. In addition, dark incubation induced white-yellow shoots.

Shoot regeneration from stored shoots at various temperatures is shown in Fig. 2. The highest (100%) regeneration rate was obtained for the shoots stored at 5°C. The shoots stored under light conditions regenerated shoots more vigorously than those stored under dark conditions (Plate 3). Some of the regenerated shoots displayed vitrification, but the frequency was independent of the temperatures during the storage period. The number of normal regenerated shoots without vitrification was also the largest in the shoots stored at 5°C (Fig. 2).

A high shoot regeneration rate was obtained in shoots stored at 5°C for a total of 2 years, provided that shoot tips 8–10 mm were once excised after 1 year of storage and then exposed to 25°C for 40 days in order to obtain intermediate shoot growth prior to re-storage at 5°C for the next year (Table 1).

*P. communis* (cv. Winter Nelis) shoots could be stored at 5°C for 64 weeks with 100% survival and 100% shoot regeneration. Unlike Senryo, the shoots of Winter Nelis elongated gradually after 32 weeks regardless of light conditions during the storage period. However, the shoots stored under light

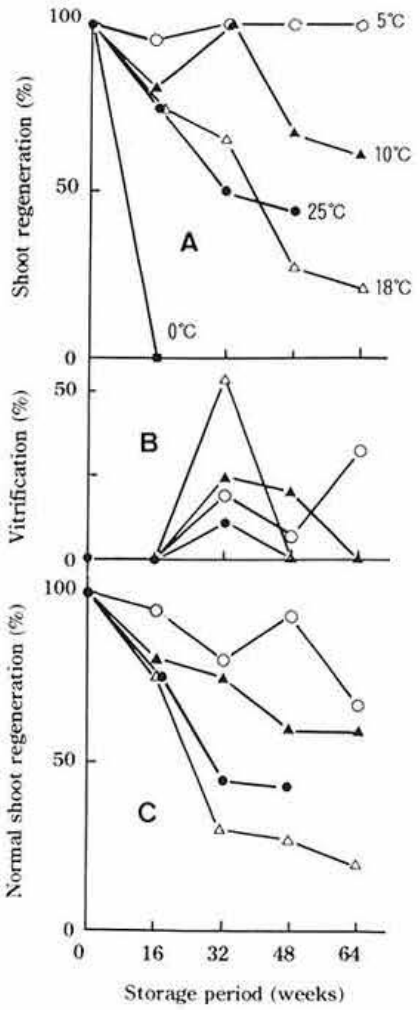


Fig. 2. Shoot regeneration from shoots of Senryo stored at different temperatures for 16, 32, 48, and 64 weeks

A: Shoot regeneration (%) (No. of shoots regenerating shoots after storage/No. of shoots stored × 100)

B: Frequency of vitrification (%) (No. of vitrified shoots/No. of regenerated shoots × 100)

C: Normal shoot regeneration (%) (No. of shoots regenerating normal shoots without vitrification/No. of stored shoots × 100).

Shoots were regenerated on MS medium containing 2.5% sucrose and 2.5 mg/l BA.

■ (0°C), ○ (5°C), ▲(10°C), △(18°C), ● (25°C).

**Table 1. Effect of intermediate shoot regeneration on improvement of survival and regeneration in Senryo shoots stored at 5°C for 2 years**

| Storage at 5°C for 2 years | Survival (%) | Regeneration (%) | No. of regenerated shoots |
|---|---|---|---|
| Without intermediate shoot regeneration | 66.7 | 12.5 | 0.4 ± 1.1 |
| With intermediate shoot regeneration* | 95.8 | 91.7 | 2.3 ± 1.5 |

* Shoots were cultured on MS + 2.5 mg/l BA for 40 days at the end of 1 year storage and then excised shoot tips were re-stored for 1 year.

conditions regenerated a larger number of shoots after 64 weeks of storage than those stored under dark conditions (data not shown).

*2) Experiment 2*

Data relating to the survival, shoot growth and shoot regeneration of the stored shoots on various kinds of slow growth media are shown in Table 2. Although shoots stored merely on the control medium could survive at 5°C for 2 years at a rate of 26.7%, the use of media supplemented with 1 mg/l ABA, 1 mg/l PP333, 1 mg/l S-327D, 2% agar, 10% sucrose or medium with 1/4 strength inorganic salts led to higher survival and shoot regeneration rates than the control medium. Shoots on the control medium gradually elongated and eventually reached an average length of 35.7 mm after 2 years of storage. Similar shoot elongation was also observed on a medium containing 2% agar or 10% sucrose. ABA slightly reduced shoot growth, but considerable inhibition of shoot elongation was achieved only when growth retardants (Paclobutrazol and S-327D) were added to the medium. These growth retardants produced rosette type shoots sometimes associated with leaf chlorosis. However, when the rosette shoots that remained green during the storage period were transferred to the regeneration medium, they normally resumed shoot growth and developed a rather larger number of shoots than those stored on the control medium (data not shown). In a medium without sucrose, shoots did not survive at 5°C even if illumination was provided; hence the shoots were not photoautotrophic at this temperature.

*3) Practical storage of pear germplasm by minimal growth method*

The storage method developed for Senryo and Winter Nelis was applied to other pear accessions for practical use of the minimal growth method for germplasm storage. There was no reduction in the survival rate for the first 6 months in all of the accessions, after which the survival rate gradually declined in *P. serotina* accessions, while it did only slightly in *P. communis* (Table 3).

## Discussion

The results obtained in this study showed that

**Table 2. Survival and shoot regeneration in *Pyrus serotina* Senryo shoots stored at 5°C on modified MS medium for 2 years**

| Medium | Survival (%) | Shoot length after storage (mm ± SD) | Regeneration (%) | Vitrification (%) |
|---|---|---|---|---|
| Control* | 26.7 | 38.0 ± 5.7 | 26.7 | 0 |
| BA 0 mg/l | 26.7 | 17.3 ± 5.7 | 12.1 | 0 |
| BA 25 mg/l | 20.0 | 9.0 ± 1.1 | 6.7 | 0 |
| ABA 1 mg/l | 70.0 | 27.0 ± 5.7 | 11.9 | 0 |
| PP333 1 mg/l | 70.0 | 9.6 ± 2.4 | 60.0 | 0 |
| S327D 1 mg/l | 60.0 | 8.1 ± 1.5 | 40.0 | 0 |
| Agar 2% | 73.3 | 35.8 ± 11.9 | 63.3 | 5.2 |
| Sucrose 0% | 0.0 | 0.0 ± 0.0 | 0.0 | 0 |
| Sucrose 10% | 80.0 | 35.8 ± 13.8 | 63.3 | 0 |
| × 1/4 MS | 80.0 | 23.5 ± 10.4 | 80.0 | 12.5 |

Shoots were regenerated on MS medium with 2.5 mg/l BA for 40 days.
*MS + 2.5% sucrose + 1 mg/l BA + 0.8% agar.

**Table 3. Survival of pear shoots grown *in vitro* after 2-year storage at 5°C**

| *Pyrus* species | No. of accessions cultured | Duration of storage | | |
|---|---|---|---|---|
| | | 6 months | 1 year | 2 years |
| *P. communis* | 90 | 90 (100%) | 88 (98%) | 84 (93%) |
| *P. serotina* | 73 | 73 (100%) | 50 (68%) | 23 (32%) |
| *P. bretschneideri* | 16 | 16 (100%) | 14 (88%) | 9 (56%) |
| Other *Pyrus* species | 48 | 48 (100%) | 39 (81%) | 20 (42%) |
| Total | 227 | 227 (100%) | 191 (84%) | 136 (60%) |

*Pyrus* spp. shoots stored at 5°C could survive and regenerate shoots at least for 1 year without subcultures. The temperature of 5°C was critical for minimal growth of pear shoots, because they died at 0°C in a short time and the survival rate declined much earlier at 10°C than at 5°C. Regarding the effect of the temperature, it was interesting to note that the shoots grown at 18°C degenerated earlier than those grown at 25°C, presumably due to the physiological imbalance between growth and respiration at the intermediate temperature of 18°C. Light conditions during storage also affected both the survival rate and regrowth of the shoots; light was found to play an important role(s) in maintaining the viability under such a low temperature. Moriguchi et al. (1990)[6] reported that European pear (*P. communis*) shoots survived at 5°C for 12 months, whereas none of the 3 cultivars of Japanese pear (*P. pyrifolia*) survived at any temperatures of 5, 10, and 15°C for 12 months. In our study, 75% of the cultivars of Japanese pear survived at 5°C (Table 3). The discrepancies between the results may be due to the genotypes as well as the concentration of BA, because Moriguchi et al. (1990)[6] used BA at 1 $\mu$M that was less than 1/10 of the concentration used in this study. Genotypic variations in low temperature storage have been observed in grape[1], apple[13], and banana[12].

Some modifications of the medium components by the addition of growth retardants or varying the concentration of sucrose (10%) or inorganic salts (1/4 strength MS) were effective to obtain higher survival rates of pear shoots that were stored for 2 years. Positive effects of the increase and decrease of the sucrose concentration on the improvement of survival were recognized in cold storage of Japanese persimmon shoot cultures[3] and storage at 20°C of *Coffea* shoots[2], respectively. Pear shoots could not survive on medium without sucrose. Similar results showing that a medium without sucrose induced low plant vigor after storage were obtained in pineapple[14].

The inhibition of shoot growth during cold storage depended on the medium conditions. However, the extent of reduction of the shoot growth after storage was not related to the survival rate. For example, although the increase of the agar concentration to 2% was effective for increasing the survival rate, this condition did not inhibit shoot elongation, suggesting that there is a suitable level of reduced metabolic activities of plant materials for long term storage.

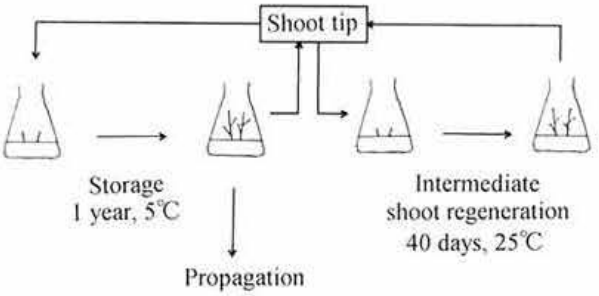


Fig. 3. A scheme for 2-year storage of *Pyrus* shoots with intermediate shoot regeneration at the end of 1 year after storage

As a simpler method, the use of MS medium with 1 mg/l BA was suitable for the storage of pear shoots for 1 year. In this case, more prolonged storage could be achieved by intermediate subculture of the stored shoots at the end of 1 year, to obtain healthy shoots for storage in the following year as well as for further shoot proliferation. Thus, a recycling system of storage of 1 year could be proposed (Fig. 3). The system was considered to be functional for a large part of pear accessions based on the results shown in Table 3, in which 98% of *P. communis* and 75% of *P. serotina* and *P. bretschneideri* accessions survived after 1 year of storage.

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