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Effects of non-frozen cold storage on the growth, organogenesis and secondary metabolism of callus cultures

N. HIRAOKA and T. KODAMA

Niigata College of Pharmacy, 5829 Kamishinei-cho, Niigata 950-21, Japan

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Abstract. Callus tissues derived from chilling-tolerant herbaceous plant, *Atractylodes lancea, Atropa belladonna, Bupleurum falcatum, Dioscorea tokoro, Lithospermum erythrorhizon* and *Phytolacca americana* could be cold-stored at 4°C for three months or more, whereas those from chilling-sensitive herbaceous plants such as *Datura innoxia* and *Perilla frutescens* var. *crispa* and a deciduous tree, *Mallotus japonicus*, could not survive after cold storage for two to three months. Tobacco callus cultures could be stored at 4°C for two or four months depending on a callus strain. The effect of cold storage on secondary metabolite production varied. Nicotine and betalain production suffered from cold storage of tobacco and *Phytolacca americana* callus cultures, respectively. However, production of anthocyanin in cultures of *Mallotus japonicus* and *Bupleurum falcatum* and shikonin derivatives in *Lithospermum erythrorhizon* callus was affected very little. Root-forming ability was retained for more than one year in cold-stored callus tissues of *Bupleurum falcatum*, while the control callus tissues maintained at 25°C completely lost the organogenetic ability six months after the first subculture.

Introduction

Plant cells cultured in vitro are generally maintained by periodically transferring them to fresh medium. Genetic, physiological or biochemical variations are often observed during serial subculture [17, 20]. To diminish such variations and to save expense and time for routine culture maintenance, preservation of cells in the inactive state by various methods has been attempted [6]. Though freeze-preservation has been most widely studied for this purpose, it requires sophisticated techniques and is not yet applicable to many plant species.

Non-frozen cold storage of cultured plant cells could be an alternative method for certain species for which freeze-preservation is not appropriate. Although there are some reports on non-frozen cold storage of callus cultures [2, 4, 7, 9, 11], the experiments in those studies are not extensive either in the duration of cold storage except one case [11] and in the number of plant species employed. In the case of freeze-preservation of cultured plant cells, rapidly growing suspension cultures are recognized to be the most suitable materials [6]. This suggests that also in non-frozen cold storage of callus cultures growth phase of callus might be an important factor affecting the viability of stored materials. Very little is known about the effect of cold

storage on the secondary metabolism in callus cultures. Jones and Veliky [8] briefly reported that immobilized carrot cells kept at 4 °C for two months retained the ability to transform digitoxigenin to periplogenin. Generally, organogenetic potential of the callus is lost with successive subculturing. The reduction of cell division is known as an effective method to retain the potential for a longer period [11]. One of the simplest methods of slowing cell division is to keep the cells at low temperatures.

Taking account of the factors mentioned above, we carried out this study to determine how long callus tissues can be stored in a refrigerator without adversely affecting the potential to produce secondary metabolites, organogenesis or growth.

Materials and methods

Tissue culture

The basal medium used for callus cultures here was Linsmaier-Skoog's one [10] containing 3% sucrose and 1% agar. The pH of medium was adjusted to 5.6 with 1 *N* NaOH before autoclaving at 120°C for 15 min. For experiments with all cultures except those from tobacco, a piece of callus (averaging 0.1 g fresh weight) was aseptically inoculated in each tube (18 × 180 mm) containing 10 ml of medium. With tobacco callus cultures four pieces of callus (each ca. 0.1 g fresh weight) were planted in each 100-ml Erlenmeyer flask containing 30 ml of medium. Plant growth regulators in the media were $10^{-6}M$ naphthalenacetic acid for *Atropa belladonna* L. (Solanaceae), $10^{-6}M$ indole-3-acetic acid (IAA) and $10^{-5}M$ kinetin for *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae), $10^{-5}M$ IAA and $10^{-6}M$ kinetin for *Nicotiana tabacum* L. 'Bright Yellow', $10^{-6}M$ 2,4-dichlorophenoxyacetic acid (2,4-D) and $10^{-6}M$ kinetin for *Bupleurum falcatum* L. (Umbelliferae) and $10^{-6}M$ 2,4-D for *Atractylodes lancea* DC. (Compositae), *Datura innoxia* Mill. (Solanaceae), *Dioscorea tokoro* Makino (Dioscoreaceae), *Mallotus japonicus* (Thunb.) Muell. Arg. (Euphorbiaceae), *Perilla frutescens* (L.) Britton var. *crispa* (Thunb.) (Labiatae) and *Phytolacca americana* L. (Phytolaccaceae), respectively. Callus tissues of *Bupleurum*, *Mallotus* and *Phytolacca* were grown at 25 ° ± 3 °C under 16 h/day of light from two cool-white fluorescent tubes (40 W) maintained at a distance of 20 cm from the cultures, whereas the others were grown in the dark at 25 ° ± 1 °C. Subcultures were performed at intervals of 4–5 weeks. Silicone sponge plugs were used throughout the experiments.

Callus cultures, which had been incubated at 25 °C for one month, were held in a refrigerator at 4 ° ± 3 °C for indicated periods. Temperature differences are referring to time but not to place. After the cold storage, they were incubated at 25 °C on fresh media for 1, 1.5, or 2 months. The growth on the basis of fresh weight of callus was compared with that of the control

cultures grown for one month at 25 °C. All the growth data are the average of five replicates.

Experiments on organogenesis were carried out with another strain of *Bupleurum falcatum* callus tissues, which were newly induced from the leaf and maintained in the dark at 25 °C on the basal medium containing $10^{-6}M$ 2,4-D. For the induction of roots, the callus tissue (averaging 1.3 g fresh weight) was transferred into a 100-ml Erlenmeyer flask containing 20 ml of liquid Linsmaier-Skoog medium supplemented with $10^{-7}M$ IAA, $10^{-5}M$ kinetin and 3% sucrose, and incubated for 7 weeks in the dark on a reciprocal shaker (90 rpm, 70 mm stroke). The root-forming ability is expressed as the percentage of cultures forming roots.

Analysis of secondary metabolites

The betalain content of *Phytolacca* callus was determined by the following modification of the method of Woodhead and Swain [22]. Callus tissues were homogenized in a mortar with 5–20 ml of $1M$ acetate buffer, pH 4.5, and extracted for 1 h at 5 °C. The homogenate was centrifuged at $1100g$ for 12 min and the turbid supernatant clarified by passing it through a membrane filter (0.45 μm pore size). The absorbance of the clear solution was measured at 540 nm. The pigment content is given as the optical density of the solution containing the extract from 1 g fresh weight of callus in 10 ml.

Anthocyanin content was determined in the following way. Callus tissues of *Mallotus* and *Bupleurum* were homogenized with 1% HCl in methanol and extracted for 1 h at room temperature. The absorbance of clear solution obtained by centrifugation at $1100g$ for 10 min was measured at 535 nm. Anthocyanin content was shown by the same unit described for betalains.

Shikonin derivatives in *Lithospermum* callus were measured by the method of Mizukami et al. [13], and the content of shikonin derivatives was given as mg shikonin per fresh weight (g) of callus tissue.

For the quantitative analysis of nicotine in tobacco callus tissue, it was dried in a hot air oven at 60 °C for longer than 8 h to reduce the volume of materials because of small capacity of a steam-distillation apparatus. Nicotine content in tobacco callus was determined as previously described [19], and expressed in terms of the percentage of nicotine on the basis of dry weight of callus tissues. All the data on the quantitative analysis of secondary products are the average value of five replicates.

Chromatographic analyses of tobacco alkaloids, anthocyanins, betalains and shikonin derivatives were carried out according to the methods of Tabata and Hiraoka [19], Harborne [5], Adachi and Ootani [1], and Tabata et al. [18], respectively.

Results

Growth and secondary metabolite production of callus cultures after cold storage

The first experiment was done to determine how long callus cultures derived from six species of dicotyledonous plants could be preserved in a refrigerator without serious deterioration. Callus tissues taken out of a refrigerator had a long lag phase. The weight increase was less than 50% of that of control after one-month incubation at 25 °C. Fresh weight of the cold-treated callus tissue increased markedly between 30th and 60th day of incubation. Therefore, the growth of the cold-stored cultures was measured after two months of growth whereas that of the control cultures was measured after one month (the usual transfer interval of stock cultures). Figure 1 shows that there is a big difference in low temperature tolerance among callus strains. The callus cultures of *Mallotus,* tobacco (strain T142) and *Dioscorea* could tolerate storage for one, two and four months, respectively. *Bupleurum, Lithospermum* and *Phytolacca* callus cultures showed a growth rate of more than 50% of that of the control culture even after six months of cold storage.

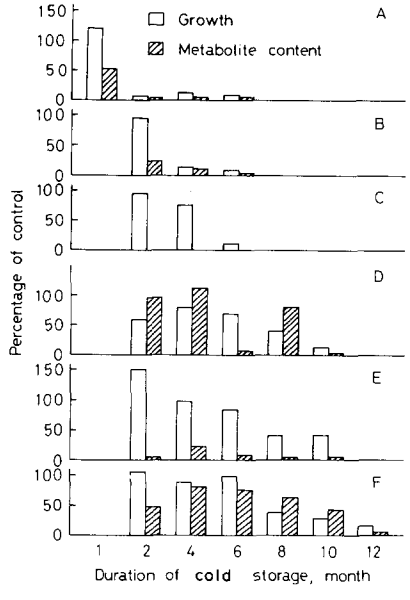

Figure 1. Growth and secondary metabolite content of callus cultures compared with the control culture after storage at 4 °C. Callus tissues were incubated at 25 °C for 2 months after cold storage for indicated periods. The control culture was grown for 1 month. Plant species and metabolites analyzed: A, *Mallotus japonicus,* anthocyanins; B, *Nicotiana tabacum* 'Bright Yellow' (strain T142), nicotine; C, *Dioscorea tokoro*; D, *Lithospermum erythrorhizon,* shikonin derivatives; E, *Phytolacca americana,* betalains; F, *Bupleurum falcatum,* anthocyanins. All the treatments were replicated with five tubes. Experiments were repeated twice with similar results.

Table 1. Growth of callus cultures after cold storage. One-month-old callus tissues were stored at 4 °C for 0 (control), 2 or 3 months. After the storage, they were transferred to fresh media and grown at 25 °C for 2 months. The control cultures were grown for 1 month at 25 °C.

Origin of callus culture		Duration of cold storage (month)		
Systematic name	Organ	0	2	3
Atractylodes lancea	Leaf	+++	+++	+++
Atropa belladonna	Leaf	++++	++++	++++
Datura innoxia	Hypocotyl	++++	++	−
	Root	++++	+++	−
	Stem	++++	+	−
Perilla frutescens var. *crispa*	Leaf	+++	+	−

−, +, ++, +++, ++++ = degree of callus growth

The decrease in the content of secondary metabolite in *Bupleurum, Lithospermum* and *Mallotus* callus cultures having undergone prolonged period of cold storage followed more or less the decrease of callus growth. The nicotine content in tobacco callus and the betalain content in *Phytolacca* callus dropped markedly after two months of cold storage, even though the calli grew well. By chromatographic analysis, we could hardly detect differences between extracts prepared from cold-stored callus cultures and those of the corresponding control cultures, an exception being the T142 tobacco callus. The relative amount of each alkaloid changed in T142 callus which was grown for two months at 25 °C after storage at 4 °C: decrease of nicotine and increase of an unidentified alkaloid of low Rf-value. However, this change in the alkaloid pattern was not consistent: some of the cold-treated calli showed normal alkaloid pattern when they were grown at 25 °C for two months.

Bupleurum, Mallotus and *Phytolacca* callus tissues have been maintained under light condition and have characteristic colors because of their constituents, anthocyanins or betalains. They decolored gradually during storage at 4 °C. The *Bupleurum* and *Mallotus* callus cultures quickly recovered their ability to accumulate the anthocyanins when they were grown at 25 °C in the light but the *Phytolacca* did not.

In attempting to determine the ability of callus strains of other plant species to tolerate cold storage, we conducted an experiment with callus cultures of *Atractylodes lancea, Atropa belladonna, Datura innoxia* and *Perilla frutescens* var. *crispa* (Table 1). The first two strains originating chilling-tolerant plants could tolerate cold storage for three months, whereas the others deriving from chilling-sensitive plants could not.

Figure 2 shows the time course of growth and nicotine production in T14, another strain of tobacco callus cultures [14]. The calli in exponential growth phase (two weeks of incubation) and in stationary phase (five weeks of incubation) were stored at 4 °C. After withdrawal at intervals of two months, they were grown on fresh media for two months at 25 °C. Figure 3

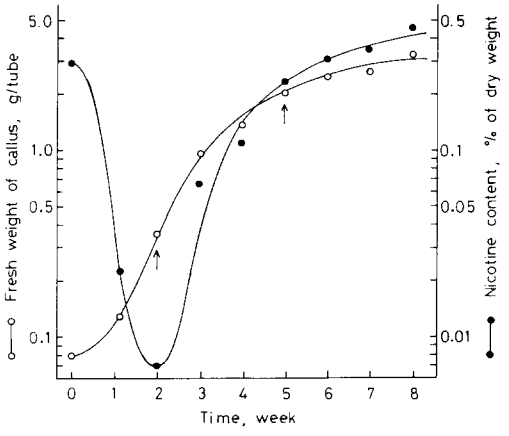

Figure 2. Time course of growth and nicotine production in tobacco tissue culture (strain T14) grown in the dark on Linsmaier and Skoog's medium containing $10^{-5}M$ IAA and $10^{-6}M$ kinetin. Arrows: callus cultures at these growth phases were cold-stored. Each point represents the mean from five replicates.

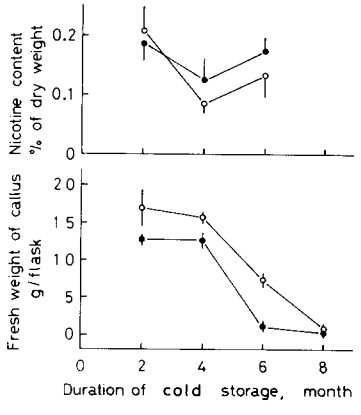

Figure 3. Relationship between the growth stage and cold tolerance in tobacco callus cultures (strain T14). Callus tissues preincubated for 2 (○) or 5 (●) weeks at 25 °C were stored at 4 °C for indicated periods. After cold storage, they were transferred to fresh media and incubated for 2 months at 25 °C. Each point represents the mean with standard error from five replicates.

shows that there is no difference in nicotine content between the two callus cultures. However, a difference was observed in growth. We could store rapidly growing calli for six months at 4 °C but could only store the calli in stationary phase for four months. Similarly, *Dioscorea* callus tissues in exponential growth phase (three weeks of incubation) and in stationary phase (six weeks of incubation) were cold-stored and the growth of callus tissues was measured in the same way as in tobacco callus cultures (Figure 4). There was no differences in growth between exponential and stationary phase

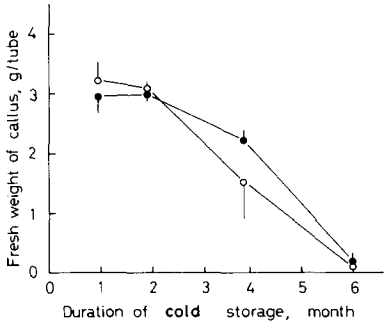

Figure 4. Relationship between growth stage and cold tolerance in *Dioscorea tokoro* callus cultures. Callus tissues preincubated at 25 °C for 3 (○) or 6 (●) weeks were preserved at 4 °C for various periods. Then, they were transferred to fresh media and incubated at 25 °C for 8 weeks. Each point represents the mean with standard error from five replicates.

cultures of *Dioscorea tokoro* irrespective of the duration of cold storage for up to six months.

Organogenic ability of cold-stored callus

The *Bupleurum falcatum* callus maintained at 25 °C can differentiate roots during the first several subculture passages. But it loses the ability to differentiate roots by about six months after the first transfer. On the other hand, when we stored the newly induced callus tissues at 4 °C for 14 months, they retained the root-forming ability without any marked decrease (Figure 5).

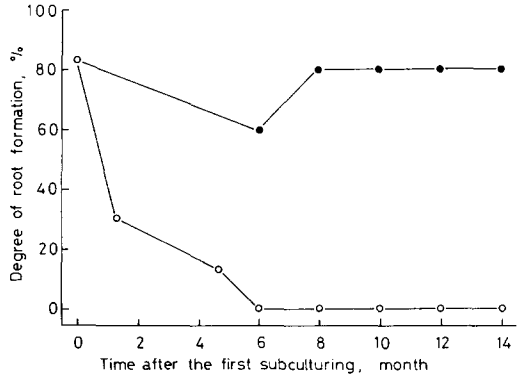

Figure 5. Retention of adventitious root differentiation ability by *Bupleurum falcatum* callus cultures during cold storage. The newly induced callus tissues were divided into two groups. One was preserved at 4 °C for up to 14 months (●). The other was subcultured at one-month intervals at 25 °C (○). A portion of calli was withdrawn from each stock culture and transferred into the root-differentiating medium. At least five cultures were used to calculate the degree of root differentiation which was expressed as the percentage of cultures forming roots.

Discussion

Minimal-growth storage of cultured plant cells or organs has been attempted by many workers to extend the subculture intervals and to minimize genetic variations during successive subculturing. Storage of shoot tips [6] or shoot culture [3, 12] at reduced temperatures for at least 10 months has been successful in some plants. However, they have been few studies on the cold storage of callus cultures. The present study with 13 callus strains exemplify wide variations in possible cold-storage periods, e.g., one month for *Mallotus* and more than six months for *Bupleurum* and *Phytolacca.* All the callus strains derived from chilling-sensitive plants, namely *Datura,* tobacco (except T14 strain) and *Perilla* were damaged by three months of storage at 4 °C. On the other hand, all the calli from chilling-resistant herbaceous plants, *Atractylodes, Atropa, Bupleurum, Dioscorea* and *Lithospermum,* could be cold-stored for three months or more. There is good correlation between the duration of storage at 4 °C of a callus culture and chilling sensitivity of the original plant as for the herbaceous plant materials used here. This correlation cannot be extended to calli from trees because only one has been examined here.

Cold storage affects not only callus growth but also capacity of callus cultures to accumulate secondary products such as nicotine in T142 cultures. Although T14 and T142 strains are clones of single-cell origin derived from a common stock callus culture and have a similar alkaloid composition under the usual culture conditions, a change in alkaloid pattern after cold storage of callus for more than two months could be found only with T142. The reason for this is not clear, though.

While storage at 4 °C seems possible for callus cultures of chilling-resistant herbaceous plant, the use of this storage method to protect calli producing secondary products does not appear to be applicable in all cases and would need to be evaluated.

Plant tissue cultures commonly lose their potential for organogenesis during prolonged periods of subculture. Reinert and Backs [15] succeeded in regulating and maintaining the totipotency of carrot cells over prolonged periods. Organonenetic ability of plant tissue culture has been maintained by minimal growth medium [16] or by freeze-preservation [6, 21]. Few workers, however, used a chilling method for this purpose. Meyer-Teuter and Reinert [11] maintained embryogenesis in slowly growing carrot cells cultured at 13 °C or at 5 °C. The present results with *Bupleurum falcatum* callus adds another demonstration of retained potential for organogenesis during non-frozen cold storage (Figure 5).

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