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Application of "CryoSeeds" in the Cryopreservation of Cultured Plant Cells and Tissues.

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In cryopreservation of living cells and tissues, artificial ice seeding after cooling the suspending medium slightly below the freezing point is an invaluable procedure to avoid intracellular freezing[1)] and to minimize thermal damage to cells from the large output of latent heat[2)]. Uniformity of temperatures at which suspending media begin to freeze is necessary to maintain an exact cooling rate during freezing. This is achieved in a controlled freezing apparatus ("program freezer"), by such special means as mechanical vibration of sample tubes, abrupt cooling for a short time by blowing liquid nitrogen into the cooling chamber (thermal method), or thermoelectric refrigeration utilizing Peltier's effect. However when simple freezing methods are employed as described previously[3-5)], accomplishing ice seeding at the desired temperatures is a difficult problem.

Very recently an ice nucleating agent termed "CryoSeeds" has been produced by Cell Systems Limited (Cambridge, UK). In the present study, we describe the application of this agent to the cryopreservation of cultured plant cells and tissues.

CryoSeeds are spherical beads 0. 15-0. 5 mm in diâmeter made of an acrylic polymer coated with Xygon. When they were added to a cryoprotective solution composed of 5 % (V/V) DMSO and 10 % (W/V) glucose in Murashige-Skoog's medium[6)] (a solution which has been used previously in the cryopreservation of callus tissues and protoplasts[7)]) and the solution was cooled slowly to below the freezing point (-3.3°C$=$1780 mOsm/kg), freezing (ice nucleation) of the solution initiated at around -6°C. Without these beads the solution supercooled to below -10°C, sometimes to about -16.5°C (**Fig. 1**).

The average ice nucleation temperatures with and without CryoSeeds were -5.9°C and -13.8°C, respectively. As shown in **Fig. 2**, ice nucleations were observed in a broad temperature range from -8 to -16.5°C when freezing without CryoSeeds (**Fig. 2**). This fluctuation is due to the fact that ice nucleation in solutions without any external nucleator is subject to a statistical event[8)]. In the freezing of cells and tissues, this fluctuation may result in a shift of the freezing curve to a lower temperature range depending upon the degree of supercooling of the solution. As a result, final freezing temperatures beyond a certain time period may vary greatly among samples during controlled freezing (prefreezing) by the two-step freezing method. Our previous studies[9,10)] have shown that the final freezing temperature of samples prior to immersion in liquid nitrogen (LN) is an important parameter affecting cell survival after cryopreservation by the two-step freezing method. Thus, the fluctuation of ice nucleation temperature as well as the incidence of intracellular freezing[1)] and thermal damage by latent heat[2)] all probably affect survival of samples treated by freezing.

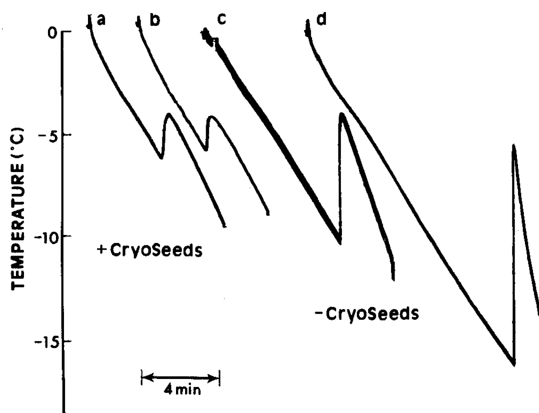

Fig. 1 Freezing curves of the cryoprotective solution (Murashige-Skoog's medium containing 5 % DMSO and 10 % glucose) with (a, b) and without (c, d) CryoSeeds.

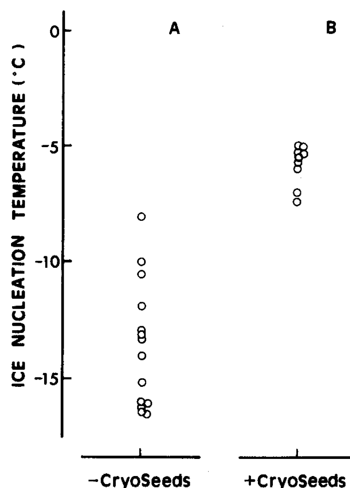

Fig. 2 Ice nucleation temperatures of the cryoprotective solution (Murashige-Skoog's medium containing 5 % DMSO and 10 % glucose) with (B) or without (A) CryoSeeds.

On the other hand, in freezing with CryoSeeds, ice nucleation temperatures centered around $-6\pm$ 1°C (**Fig. 2**). These results suggest that the possibility of the survival fluctuation or survival decrease arising from the causes described above is least in the freezing with CryoSeeds. In the cryopreservation of *Aegilops squarrosa* cells, an increase of about 15 per cent cell survival was observed with CryoSeeds (**Table 1.**). Thus, the survival decrease of about 15 per cent seen in the freezing without CryoSeeds may be considered to have arisen from such causes. However, further studies are needed to clarify which cause(s) mainly contribute to survival decrease.

The effect of CryoSeeds on the growth of cultured plant cells and tissues was examined. Callus tissues of soybean (*Glycine max*) were cultured on agar medium with 5-10 beads of CryoSeeds. As shown in **Tabl 2.** little or no effect on the growth of the tissues and no visual change in color or surface texture were observed. Very similar results were obtained in a suspension culture of *A. squarrosa* and callus cultures of *Marchantia polymorpha* and *Daucus carota* (data not shown).

CryoSeeds showed highest ice nucleation activity when they were placed on the wetted inner surface of a sample tube near the surface of the sample solution. If the beads were suspended or floated on the sample solution, the solution sometimes supercooled to -10 °C. CryoSeeds can be stored at room temperature but should be kept without shaking to avoid removal of Xygon which is the ice nucleating substance coated on the surface of the beads, If a large portion of Xygon is removed from the bead surface and adheres to the surface of a glass vial, it can be scraped off as powder with a small spatula. It was found that this powder showed very high ice nucleation activity when added directly to the sample solution.

CryoSeeds were packed in a glass vial after being sterilized by irradiaton. As shown in **Table 2,** no microbal contamination was observed in callus cultures of soybean which were handled under usual sterile conditions. The activity of CryoSeeds was completely lost after being autoclaved or treated with 70 % ethyl alcohol.

Table 1. Survival of suspension-cultured wheat (*Aegilops squarrosa*) cells after cryopreservation in LN with and without CryoSeeds.

Freezing*	TTC reduction** (O. D. $_{at\ 530}$/tube)	Survival (%)
Control (no freezing)	2. 79	100
Without CryoSeeds	1. 31±0. 32***	46. 8±11. 7***
With CryoSeeds	1. 73±0. 24	62. 0±8. 6

* Cells treated with cryoprotective solution (5 % DMSO plus 10 % glucose in Murashige-Skoog's medium) were slowly (1. 3°C/min) frozen to −30°C and then immersed in LN. The frozen cells were thawed rapidly in water at 40°C.

** TTC reduction test was carried out according to the method of Steponkus and Lanphear (1967)[11].

*** Average±S. D. (n=5)

Table 2. Effect of CryoSeeds on the growth of soybean (*Glycine max*) callus cultures.

Culture	Growth* (g fr. wt./tube)	Number of tubes contaminated/total tubes
Without Cryo-Seeds (control)	0. 66±0. 11**	0/18
With CryoSeeds	0. 67±0. 14	0/30

* 0. 1 g of callus tissue was cultured on 10 m*l* of agar medium for 16 days.

** Average±S. D.

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《和文要約》

"CryoSeeds"の植物培養細胞・組織の凍結保存への応用

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氷核形成活性のあるCryoSeedsを植物培養細胞・組織の凍結保存に応用した．CryoSeedsを含まない，Murashige-Skoogの培地にDMSOとグルコースを添加した凍害防御溶液は過冷却し，−8～−16.5℃の広い範囲にわたり凍結が起こった．CryoSeedsを含む凍害防御溶液では，いずれも−6℃付近で凍結が開始した．タルホコムギ（*Aegilops squarrosa*）の懸濁培養細胞の液体窒素中の凍結保存では，CryoSeedsを加えて凍結した方が，加えずに凍結した場合に比べて約15％の生存率の増加があった．数種のカルスや懸濁培養細胞の増殖は，CryoSeedsの添加によってほとんど影響されなかった．