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Long-term Storage and Cryopreservation of Desiccated Microspore-derived Embryos in *Brassica* spp.

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Abstract

Microspore-derived embryos of *Brassica napus* were cryopreserved in liquid nitrogen (LN) after desiccation treatment. Desiccated embryos which were directly immersed in LN converted to plantlets without any decrease in their viability. In contrast, non-desiccated embryos lost their viability after cryopreservation. The longevity and viability of the desiccated embryos of *B. campestris* were examined under several storage conditions. Survival rates of the embryos were affected by temperature of storage. Lower temperature conditions prolonged their viability. Storage up to 3 years at −80°C did not affect the plant conversion ability of desiccated embryos. These results suggest that naked desiccated embryos of *Brassica* spp. could be stored for a long period at lower-temperature.

1. Introduction

Somatic embryos induced *in vitro* have a great value for the development of crop improvement and propagation as well as plant biological research. Artificial seed technology is one of the important applications of somatic embryos. Though various types of artificial seeds have been proposed [1], many problems remain to be overcome, such as coating materials, storage and plant conversion under various soil conditions.

Desiccated artificial seeds proposed by Kitto and Janick [2] are thought to be a convenient form for practical utilization. Naked desiccated embryos are the simplest form of artificial seeds. Successful induction of desiccation tolerance of somatic embryos has been reported in some plants [3-7]. We have reported that desiccation tolerance of *Brassica* spp. microspore-derived embryos was induced by exogenous application of abscisic acid (ABA) [8-10]. Naked desiccated embryos of *B. oleracea* [9] and *B. napus* [8] maintained their viability for 3 and 6 months, respectively. However, longevity of the embryos and the influence of storage conditions on embryo viability have not been elucidated. Preservation of viable desiccated embryos for prolonged periods remains an important problem for most plants. If desiccated embryos can be stored for a longer period, they could be utilized for germplasm preservation as well as artificial seeds.

The present paper describes the long-term preservation of desiccated microspore-derived embryos in *Brassica* spp. in a commercial freezer and cryopreservation by direct immersion in liquid nitrogen (LN).

2. Materials and Methods

2. 1 Plant Material

Rapeseed (*B. napus* cv. Topas) and Chinese cabbage (*B. campestris* ssp. *pekinensis* cv. Ho Mei) were used. These plants were grown in a greenhouse, and then transferred to a growth chamber under a 13/8°C day/night regime with a natural photoperiod at the beginning of bolting.

2. 2 Microspore culture

Flower buds, 3. 0-4. 0 mm and 2. 0-3. 0 mm in length, were collected from rapeseed and Chinese cabbage, respectively. Sterilization and microspore isolation were carried out as described previously [11]. After washing, the microspores of rapeseed and Chinese cabbage were suspended at a density of 3×10^4/m*l* and 5×10^4/m*l* in 1/2NLN-13 [11] and 1/2NLN-10 [10] medium, respectively. Two ml of the microspore suspension was plated in a 60×15 mm plastic petri dish. The microspores of rapeseed and Chinese cabbage were incubated in the dark at 32. 5°C for 4 days and 1 day, respectively, before incubation at 25°C.

2. 3 ABA treatment and embryo desiccation

After 14 days of culture, the medium of rapeseed and Chinese cabbage was replaced by 2 m*l* fresh 1/2NLN-13 and 1/2NLN-10 supplemented with 100 μM and 10 μM ABA, respectively. After 7 days of incubation in the dark at 25°C, late torpedo to cotyledonary stage embryos were selected by sieving through 200 μm nylon mesh, and then washed with sterile

deionized water. The embryos were transferred onto a sterile filter paper, which was moistened with a few drops of sterile deionized water, in a 60×15 mm plastic petri dish.

Embryo desiccation was performed according to the method of Takahata *et al.* [9]. Embryos were desiccated through a series of desiccators, in which the relative humidities (RH) were kept constant by saturated solutions of K_2SO_4 (RH 87%), Na_2CO_3 (80%), NaCl (70%), NH_4NO_3 (61%), $Ca(NO_3) \cdot 4H_2O$ (50%) and $K_2CO_3 \cdot 1.5H_2O$ (40%). They were transferred daily from a desiccator at higher RH to one at lower RH.

2. 4 Storage of desiccated microspore-derived embryos

After desiccation treatment, the dry embryos of rapeseed were transferred to a 1. 5 m*l* tube, immersed in LN and stored for 1, 3 and 7 days. The desiccated embryos without ABA treatment and non-desiccated embryos with and/or without 100 μM ABA treatment were also cryopreserved for 1 day.

On the other hand, the dry embryos of Chinese cabbage in 60×15 mm plastic petri dishes were stored for several periods (3 to 36 months) under various storage conditions such as room temperature (RT), a refrigerator and a freezer.

2. 5 Determination of germination and plant regeneration ability

Cryopreserved embryos of rapeseed were rewarmed by immersing the tubes in a water bath at 40°C for 2 min. The embryos were transferred to a filter paper placed on top of B5 [12] agar (0. 8%)-solidified medium containing 2% sucrose (B5-2). As an additional control, cryopreserved embryos were directly transferred to a filter paper placed on top of B5-2.

On the other hand, after storage for several periods, the filter papers with Chinese cabbage embryos were directly transferred to B5-2. The embryos were incubated at 25°C under a 16 h photoperiod (30 μmol m^{-2} s^{-1}), and then the frequency of germination and plant regeneration was examined after 1 and 4 weeks of culture, respectively.

3. Results and Discussion

After the desiccation regime, microspore-derived embryos of rapeseed and Chinese cabbage decreased in size and became yellowish and shrunken. The final water content of the embryos was approximately 10%. After rehydration of the embryos, they rapidly regained their initial size before desiccation, and those which maintained their viability turned green and developed a root within 5 days. The frequencies of germination and plant regeneration of desiccated embryos that were not stored were 90% and 89% for rapeseed and 100% and 58% for Chinese cabbage, respectively (**Tables 1 and 2**). These results are consistent with our previous ones [8, 10].

The viability of embryos after storage in LN for 1 -7 days is indicated in **Table 1**. The embryos, which were subjected to cryopreservation and rapid thawing at 40°C, germinated and converted to plants at the percentage of 61-80% and 55-74%, respectively. These values were not significantly different from those of the non-cryopreserved embryos. The cryopreserved embryos, which were thawed slowly at room temperature, showed survival ability similar to that of embryos rapidly thawed. In contrast, non-desiccated embryos lost their viability after cryopreservation.

Successes of somatic embryo cryopreservation are reported to require treatments of cryoprotectant com-

Table 1. Survival rates after storage in liquid nitrogen (LN) in microspore-derived embryos of rapeseed. Values followed by the same letter in a column are not significantly different at the 5% level, according to Ryan's multiple range test.

Treatment				No. of embryos tested	Embryo germination*2 (%)	Plant regeneration*3 (%)
ABA*1	Desiccation	LN	Rewarm			
+	+	1 day	40°C	68	67. 6±13. 8 a	67. 6± 7. 0 a
+	+	3 days	40°C	92	80. 4± 8. 9 a	73. 9±11. 5 a
+	+	7 days	40°C	193	60. 6±11. 2 a	55. 4±12. 6 ab
+	+	3 days	RT	45	91. 1± 1. 4 a	88. 9± 9. 6 a
−	−	1 day	40°C	19	0. 0± 0. 0 b	0. 0± 0. 0 b
+	−	1 day	40°C	29	0. 0± 0. 0 b	0. 0± 0. 0 b
+	+	—	—	70	90. 0± 5. 6 a	88. 6± 6. 9 a
+	−	—	—	71	93. 0±11. 1 a	87. 3± 9. 0 a
−	−	—	—	38	77. 8±17. 2 a	69. 4±16. 3 a

*1 Embryos treated with 100 μM ABA for 7 days.

*2 Examined after 1 week from rehydration of desiccated embryos.

*3 Examined after 4 weeks from rehydration of desiccated embryos.

Table 2. Effect of storage conditions for germination and plant regeneration of desiccated microspore-derived embryos in Chinese cabbage.

Condition of storage	Period of storage (months)	No. of embryos tested	Embryo germination*1 (%)	Plant regeneration*2 (%)
−80°C	3	56	75.0±6.5	62.5±7.0
	6	28	89.3±2.1	75.0±0.0
	9	32	59.4±1.8	40.6±4.7
	12	29	75.9±0.9	44.8±1.2
	18	33	72.7±6.6	42.4±12.1
	26	10	70.0	50.0
	36	25	76.0±2.9	44.0±6.8
−20°C	3	44	82.0±4.2	45.5±3.6
	6	17	70.6	64.7
	9	31	41.9±1.9	19.4±0.6
	12	32	34.3±5.7	6.2±0.4
	18	36	25.0±10.0	16.6±2.8
	26	36	41.6±1.4	13.8±0.5
	36	17	0.0	0.0
4°C	3	—	—	—
	6	14	50.0	42.9
	9	27	66.7±4.8	22.2±5.0
	12	29	6.9±0.2	0.0
	18	43	0.0	0.0
	26	14	0.0	0.0
	36	24	0.0	0.0
Room temperature	3	77	64.9±11.1	37.7±7.5
	6	13	30.8	15.4
	9	30	13.3±0.2	0.0
	12	47	0.0	0.0
	18	29	0.0	0.0
	26	31	0.0	0.0
	36	22	0.0	0.0
Room temperature	3	70	55.7±5.3	27.1±7.1
Relative humidity 87%	6	40	0.0	0.0
	9	31	0.0	0.0
	12	29	0.0	0.0
	18	12	0.0	0.0
	26	33	0.0	0.0
	36	20	0.0	0.0
No storage	0	12	100	58.3

*1 Examined 1 week after rehydration.

*2 Examined 4 weeks after rehydration.

ponent and/or prefreezing (summarized by Florin *et al.* [13]). It is known to be difficult to cryopreserve late-stage embryos [13]. On the other hand, our results indicated that embryos (late torpedo to cotyledonary stage) could survive direct immersion in LN, when they were desiccated. Similar observations have been reported in somatic embryos of melon [14] and carrot [13]. These results suggest that a desiccation method enables the simplest cooling procedure in the cryopreservation of somatic embryos. Although the desiccated embryos were cryopreserved for 7 days in the present study, it is expected that storage for a prolonged period would be possible.

To evaluate the storage ability of desiccated embryos, those of Chinese cabbage were directly stored under 5 different conditions, as shown in **Table 2**, for 3 to 36 months. Although the desiccated embryos had the ability of storage, their survival rate depended on the storage conditions (**Table 2**). Lower temperature conditions prolonged the viability of the embryos, which is consistent with the situation for true seeds. Embryos stored at −80°C maintained their viability during 36 months, and the frequencies of germination and plant regeneration of embryos did not decrease

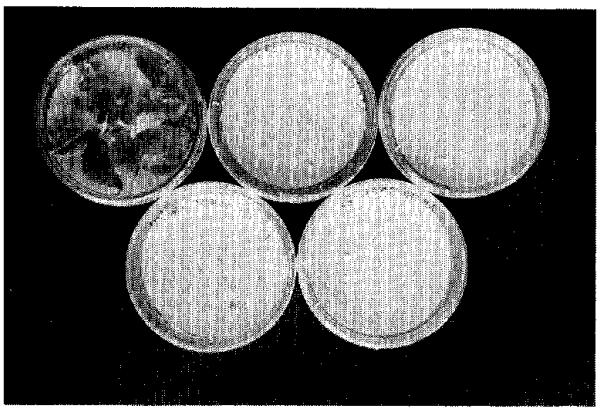

Fig. 1 Plant regeneration from desiccated embryos of Chinese cabbage stored for 36 months. Embryos were stored at RT (top left), RT (RH 87%) (top right), 4°C (bottom left), −20°C (bottom middle) and −80°C (bottom right).

after 36 months (**Fig. 1**). The regenerants from embryos stored for 36 months had the same morphology as non-stored controls. The viability of embryos which were stored at higher temperature gradually decreased with the period of preservation. Embryos stored at −20°C could survive for 26 months with 13.8% of plant regeneration, though their viability decreased with length of the storage period. Embryos stored at 4°C and RT maintained their ability of plant regeneration for 9 and 6 months, respectively. In contrast, storage at RT with 87% RH resulted in loss of plant regeneration ability within 6 months. These results indicate that the storage behavior of the desiccated embryos mimicked that of *Brassica* true seeds.

Though a storage duration study using desiccated somatic embryos showed a storage period of 1 year at RT for alfalfa [4], 6 months at RT for rapeseed [8] and 8 months at 4°C under 45% RH for carrot [13], the longevity of the desiccated embryos was not known. Our results demonstrated that desiccated embryos remained viability when stored at −80°C for 3 years, confirming that long-term preservation is feasible.

In this study, we clarified that naked desiccated embryos of *Brassica* spp. could be stored for a long period. Though storage of embryos in LN is a good method of long-term storage, it requires special equipment and periodic LN supply. On the other hand, direct storage of desiccated embryos in a commercial freezer is a simple, convenient and low-cost method for preservation of artificial seeds and for germplasm preservation. However, the maximum storage duration is under investigation.

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