DEVELOPMENT OF A WHOLE PLANT FROM A SHOOT TIP OF *ASPARAGUS OFFICINALIS* L. FROZEN DOWN TO −196°C

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Introduction

Recently, cryopreservation of genetic resources is increasing in importance as an international co-operative, biological program.

The successful development of a whole plant from an excised shoot apex after freezing down to −196°C was first reported with a carnation plant by SEIBERT and WETHERBEE[15]. After which, success in freeze-preservation of a shoot apex in liquid nitrogen were reported in strawberry[8,13] pea,[7] tomato[5], potato[4], and other some higher plant species.

In this paper, five experiments were carried out to clarify some factors contributing to the viability of a lateral shoot tip of asparagus spear frozen down to temperatures of liquid nitrogen (−196°C).

Materials and Methods

All of the experiments were carried out in January through to February, 1980, using asparagus spears (cv. Mary Washington 500) sprouting from the 5 year old plants which were transplanted to a green house from the field in November, 1979.

Small unbranched lateral shoot tips (1.5 to 2.0 mm in length) with some growing points were excised from the surface-sterilized lateral shootlets of the asparagus spears, and immersed in 0.25 ml of 3% glucose solution in a graduated spitz tube (15 mm in diameter, 115 mm in length). Then, these samples were treated by the following methods in each experiment.

Experiment I. Cold resistance of asparagus shoot tips without preculture and DMSO treatment:

The spitz tubes were plunged in an ethanol bath at 0°C, then they were

cooled down to −2.5°, −5°, −7.5° and −10°C at a rate of 0.5°C/min. After left standing at each required temperature for 2 hours, each spitz tube was rewarmed rapidly in water at 40°C, and then the sample tissues were planted on the solid medium and cultured aseptically. The medium used contained inorganic and organic substances of MURASHIGE and SKOOG's (MS) medium, 2% sucrose, 0.75% agar, 10^{-6} M IBA and 5×10^{-8} M BA. The cultures were maintained under 25°C and 16-hour illumination of 4,000 lx per day.

Experiment II. Effect of concentrations of DMSO on survival of shoot tips frozen down to −196°C:

The samples were immersed in 0.25 ml solution containing 3% glucose and 4, 8, 12, 16 and 20% DMSO (dimethyl sulfoxide) in a spitz tube. After left standing at 3°C for 2 hours, spitz tubes were plunged in an ethanol bath at 0°C, and cooled down to −10°C in 10 minutes. To induce freezing in the solution and the sample tissues, the outer surface of the tube wall touching the solution was cooled with dry ice for a few seconds. Frozen samples were successively cooled down to −40°C at a rate of 0.5°C/min, and maintained at the temperature for 10 minutes. These spitz tubes were very swiftly plunged in liquid nitrogen.

After left standing at −196°C for 30 minutes, these tubes were rewarmed rapidly in water at 40°C (rewarming rate: 500°C/min). After 30 min. of immersing of the samples in MS liquid medium to remove DMSO, they were planted on MS solid medium. The MS medium used contained 2% sucrose, 0.75% agar, 5×10^{-7} M IBA and 10^{-7} M BA. The cultures were maintained under 25°C and 16-hour illumination of 4,000 lx per day.

Experiment III. Effect of preculture with the medium containing 4% DMSO on survival of shoot tips frozen down to −196°C:

Samples were cultured on the solid medium containing the required substances for 2 days before the freezing treatment.

This was referred to as 'preculture'. The medium used in pre-culture contained inorganic and organic substances of MS medium, 3% glucose, 4% (v/v) DMSO and 0.75% agar. The culture was maintained under 25°C and 16-hour illumination of 4,000 lx per day. After pre-culture, the samples were treated with the same method as mentioned in Experiment II.

Experiment IV. Effect of prefreezing temperature on survival of shoot tips frozen down to −196°C:

Samples were immersed in 0.25 ml of solution containing 3% glucose and 16% DMSO in a spitz tube for 2 hours at 3°C; then the spitz tubes were cooled down to −10, −15, −30, −40, −50 and −60°C at a rate of 0.5°C/

min. Then, each spitz tube were plunged in liquid nitrogen for 30 minutes. After which, the samples were rewarmed rapidly and cultured aseptically by the same method of Experiment II.

Another cooling method was applied in this Experiment for trial. Namely, tissues treated with 16% DMSO solution were placed on a coverglass and the surrounding solution was removed.

Then these samples were very rapidly cooled by direct immersion in liquid nitrogen (cooling rate: 10^5°C/min) without prefreezing.

After left standing for 30 minutes at −196°C, the coverglass was immersed in the MS solution containing 3% sucrose at 40°C.

Experiment V. Effect of cooling rate in prefreezing on survival of shoot tips frozen down to −196°C:

Sample tissues were precultured for 2 days according to the method mentioned in Experiment III, followed by immersing in 16% DMSO solution in a spitz tube for 2 hours at 3°C. Spitz tubes were successively cooled down from −10°C to −40°C at five different rates (0.5, 1, 2.5 and 10°C/min). After which, they were plunged in liquid nitrogen for 30 minutes and were rewarmed rapidly in water at 40°C. The procedure of washing and planting were the same as in Experiment II.

Twenty tissues were used in each treatment in Experiment I-V.

Results and Discussion

Experiment I. Cold resistance of asparagus shoot tips without pre-culture and DMSO treatment:

Sample tissues which sustained freezing injury lost their original light green color and showed a semitransparent white color. On the 30th day in the culture after thawing, the survival rate was about 80% at −2.5°C treatment, 40% at −5°C and 7.5°C treatment and 10% at −10°C treatment, respectively (Fig. 1). From these results, it may be suggested that the meristem cells of asparagus has a high cold resistance essentially. However, it is necessary to use a cryoprotectant such as DMSO for survival of tissues cooled down to −196°C.

Experiment II. Effect of concentrations of DMSO on survival of shoot tips frozen down to −196°C:

On the effect of DMSO in DMSO solution-immersion treatment, NAG and STREET[9] and SAKAI and SUGAWARA[12] reported that 5 to 8% DMSO was essential for the survival in cultured plant cell. However, in the shoot tips, it was shown that relatively high concentrations of DMSO such as 12 to

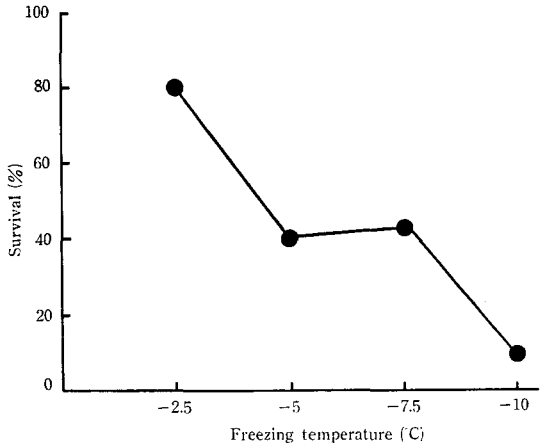

Fig. 1. Cold resistance of asparagus shoot tips without preculture and DMSO treatment.

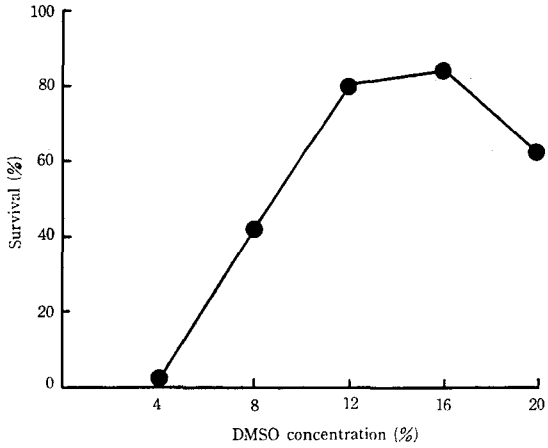

Fig. 2. Effect of DMSO concentration on survival of shoot tips immersed in liquid nitrogen following prefreezing to −40°C.

16% was effective for survival.[13,17] The same results were observed in this experiment. Namely, 12 to 16% DMSO was essential for the survival of shoot tips of asparagus, and the survival rate of the shoot tips was 80 to 82% in the presence of 12 to 16% DMSO (Fig. 2).

In DMSO treatment, 120 minutes of immersing treatment in 12-16% DMSO solution at 3°C was sufficient for a high rate of survival in asparagus shoot tips.

Experiment III. Effect of preculture with the medium containing 4% DMSO on survival of shoot tips frozen down to −196°C:

The effect of preculture on survival of shoot tips was observed in this experiment, because a high survival rate was obtained in each concentration of DMSO. Especially, in the presence of 8 to 20% of DMSO, the survival rate was 95 to 100% (Fig. 3). This fact suggests that penetration of DMSO into meristematic cells is gradually obtained during the preculture period.

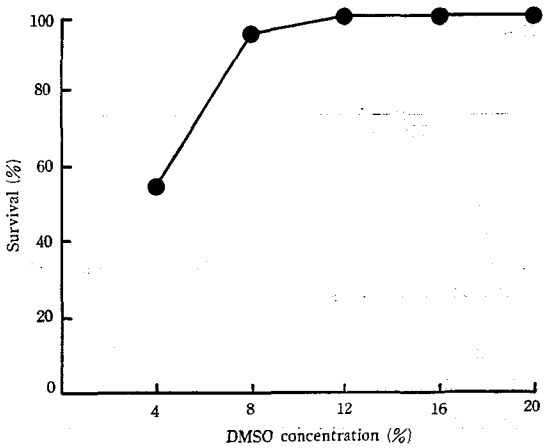

Fig. 3. Effect of DMSO concentration on survival of shoot tips immersed in liquid nitrogen following prefreezing to −40°C after 2 days pre-culture.

Experiment IV. Effect of prefreezing temperature on survival of shoot tips frozen down to −196°C:

In general, prefreezing greatly reduces the growth rate of intracellular ice crystals formed during rapid cooling and is very effective for survival.[10,11] In this experiment, the survival rate was lowest (about 30% on the 30th day after thawing) at −10°C of prefrozen temperature and it increased with the decreasing of temperatures down to −30°C, reaching about 100% (Fig. 4). In the shoot tips prefrozen to −30, −40, −50 and −60°C, survival rate was 100%.

Superrapid cooling method without prefreezing was reported in some plants[2,3,4,6,14,15,16]. In this experiment, comparatively high survival (about 65%) was observed with direct immersion in liquid nitrogen (cooling rate : 10^5°C/min). Such a superrapid cooling method will be useful for asparagus and for some other plant samples because this method is simple and does not require a long time.

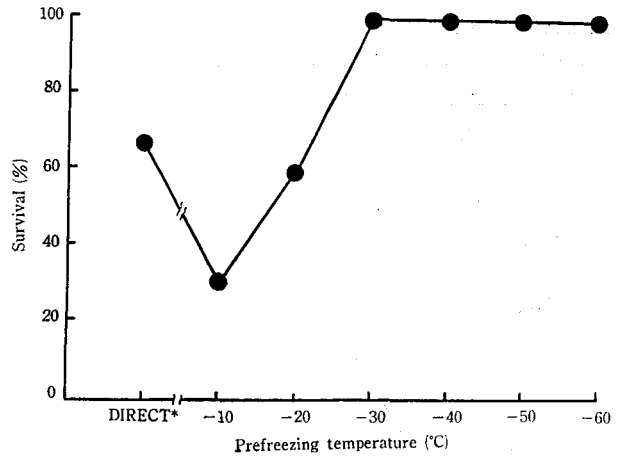

Fig. 4. Effect of prefreezing temperature on survival of shoot tips immersed in liquid nitrogen.

*: Direct immersion in liquid nitrogen using coverglass without prefreezing.

Experiment V. Effect of cooling rate in prefreezing on survival of shoot tips frozen down to −196°C:

On the cooling rate in prefreezing, 0.5-1.0°C/min was usually used for shoot tips. In this experiment, the survival rate was lowest (about 20%) at

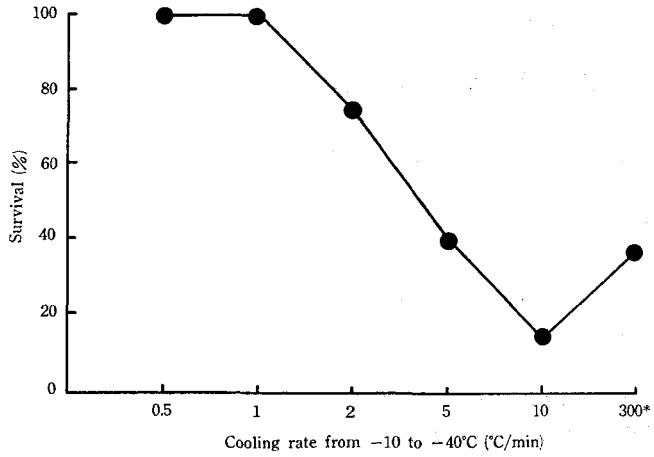

Fig. 5. Effect of cooling rate in prefreezing on survival of shoot tips immersed in liquid nitrogen.

*: Samples in the spitz tubes were cooled directly in liquid nitrogen from −10°C.

10°C/min of cooling rate and it increased with the decreasing cooling rate down to 0.5°C/min, reaching 100% (Fig. 5). This result was included in the limit reported above.

When the spitz tubes including samples were cooled directly in liquid nitrogen from −10°C, percentage of survival was about 40%.

Shoot tips that survived in this experiment grew normally on the plant-regenerating medium and developed roots after about 8 to 12-week incubation. After 6 or more months, some of these samples successfuly developed to whole plants (Fig. 6, 7). These facts suggest that it may be possible to establish a method of long-term storage in liquid nitrogen in the near future.

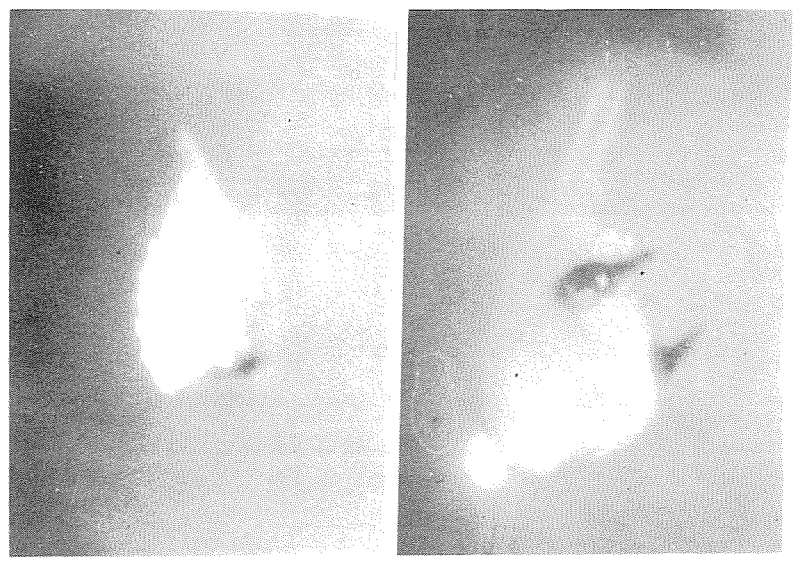

Fig. 6. Development of shoot tips on the culture medium after thawing.

A : A shoot tip planted on the culture medium immediately after thawing.

B : Development of lateral buds from a shoot tip cultured for 2 weeks after thawing.

Summary

Experiments were carried out to determine some factors contributing to the survival of lateral shoot tips of asparagus spears frozen down to the temperature of liquid nitrogen (−196°C). The results obtained are summarized as follows;

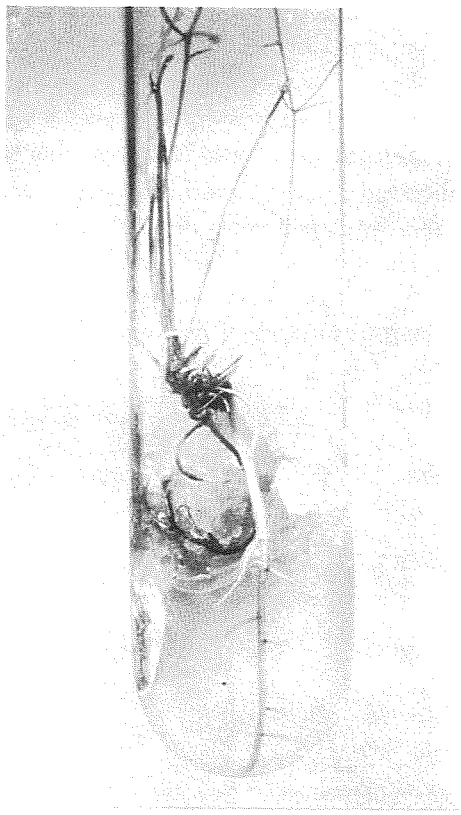

Fig. 7. A plantlet obtained from a shoot tip frozen down to −196°C.

1. Only 10% survival was obtained at −10°C in 3% glucose solution without DMSO treatment, while a high percentage of survival (80–100%) was obtained after freezing down to −196°C using DMSO as a cryoprotectant.

2. Regarding DMSO treatment, it was found that 120-minute immersion in 12 or 16% DMSO solution at 3°C was most effective for the survival of samples.

3. Two-day preculture with the solid medium containing 4% DMSO before immersing treatment in the 16% DMSO solution was also effective for the viability of samples.

4. Prefreezing treatment in the presence of 16% DMSO before liquid nitrogen treatment was effective for survival of samples, likewise. In this case, the survival rate of samples prefrozen to −30 to −40°C was 100%. In prefreezing from −10°C to −40°C, the cooling rate of 0.5°C/min showed

the highest survival (about 100%).

5. A comparatively high percentage of survival (about 65%) was obtained by immersing the samples on a coverglass in liquid nitrogen directly. In this case, the cooling rate was 10^5°C/min.

6. Shoot tips that survived in this experiment grew normally on the plant-regenerating medium and successfully developed to whole plants. These facts suggest that it may be possible to establish a method of long-term storage in liquid nitrogen in the near future.

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Literature Cited

1. ANDERSON, J. D. and MC DANIEL, R. G.: Low temperature preservation of carnation meristems. *Abst. in 1979 ASHS annual meeting. Hortscience* **14**: 423. 1979
2. BAJAJ, Y. P. S.: Clonal multiplication and cryopreservation of cassava through tissue culture. *Crop Improv.* **4**: 198–204. 1977
3. BAJAJ, Y. P. S.: Regeneration of plants from potato meristems freeze-preserved for 24 months. *Euphytica* **30**: 141–145. 1981
4. GROUT, B. W. W. and HENSHAW, G. G.: Freeze-preservation of potato shoot-tip cultures. *Ann. Bot.* **42**: 1227–1229. 1978
5. GROUT, B. W. W., WESTCOTT, R. J. and HENSHAW, G. G.: Survival of shoot meristems of tomato seedlings frozen in liquid nitrogen. *Cryobiology.* **15**: 478–483. 1978
6. GROUT, B. W. W. and HENSHAW, G. G.: Structural observations on the growth of potato shoot-tip cultures after thawing from liquid nitrogen. *Ann. Bot.* **46**: 243–248. 1980
7. KARITA, K. K., LEUNG, N. L. and GAMBORY, O. L.: Freeze-preservation of pea meristems in liquid nitrogen and subsequent plant regeneration. *Plant Sci. Lett.* **15**: 7–15. 1979
8. KARTHA, K. K., LEUNG, N. L. and PAHL, K.: Cryopreservation of strawberry meristems and mass propagation of plantlets. *J. Amer. Soc. Hort. Sci.* **105**: 481–484. 1980
9. NAG, K. K. and STREET, H. E.: Freeze preservation of cultured plant cells. I. The pretreatment phase. *Physiol. Plant.* **34**: 254–260. 1975
10. SAKAI, A.: Survival of plant tissue at super-low temperatures. IV. Cell survival with rapid cooling and rewarming. *Plant Physiol.* **41**: 1050–1054. 1966
11. SAKAI, A.: Some factors contributing to the survival of rapidly cooled plant

cells. *Cryobiology.* 8: 225–234. 1971

12. SAKAI, A. and SUGAWARA, Y.: Survival of plant germplasm in liquid nitrogen. In plant Cold Hardiness and Freezing Stress (LI, P. H. and A. SAKAI eds.) *Academic Press, N. Y.*: 345–359. 1978
13. SAKAI, A., YAMAKAWA, M., SAKATA, D., HARADA, T. and YAKUWA, T.: Development of whole plant from an excised strawberry runner apex frozen to −196°C. *Low Temp. Sci.*, **B 36**: 31–38. 1978
14. SEIBERT, M.: Shoot initiation from carnation shoot apices frozen to −196°C. *Science* **191**: 1178–1179. 1976
15. SEIBERT, M. and WETHERBEE, P. J.: Increased survival and differentiation of frozen hernaceous plant organ cultures through cold treatment. *Plant Physiol.* **59**: 1043–1046. 1977
16. TOWILL, L. E.: Solanum tuberosum: A model for studying the cryobiology of shoot-tips in the tuber-bearing Solanum species. *Plant Sci. Letters* **20**: 315–324. 1981
17. UEMURA, M. and SAKAI, A.: Survival of carnation (*Dianthus caryophyllus* L.) shoot apices frozen to the temperature of liquid nitrogen. *Plant & Cell Physiol.* **21**: 85–94. 1980