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In vitro preservation of *Asparagus officinalis*

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Abstract

A simple systems for *in vitro* storage of health asparagus germplasm was developed. High percent (90 %) of shoots cultured in a standard multiplication medium were maintained viable *in vitro* at 5 °C in darkness for 12 months. This percent was decreased to 60 % when cultures were stored for 18 months. At normal temperature, shoots and callus cultures also survived for 1 year under osmotic stress on medium containing 40 g dm^{-3} mannitol.

Additional key words: cryopreservation, mannitol, osmotic stress.

Introduction

In vitro preservation of plant cells, embryos and meristems has become an important tool for the long-term storage of germplasm as it offers several advantages over maintaining field collections. Moreover, with increasing interest in genetic engineering of plants, the preservation of cultured cells with unique attributes, in particular, genetically modified cells and plants is becoming of greater importance.

One of the principle long-term *in vitro* conservation method is cryopreservation which is generally understood as storage between -79 and -196 °C, the low extreme being the temperature of liquid nitrogen. The major advantage of storage of biological material at such temperatures is that both metabolic processes and biological deterioration are considerably slowed or even halted (*e.g.* Kartha 1981). Cryopreservation protocols were developed for approximately 40 tropical plant species (Engelmann 1991). However the freezing method needs controlled freezing equipment and a complicated cryoprotective procedure. *In vitro* storing by slow growth is achieved by modifications of the culture medium (Withers 1987), and/or low temperature (Withers 1991). In asparagus, plantlets were succesfuly stored for 24 weeks at 7 °C (IBPGR 1987), callus cultures remained viable after 15 - 16 months of storage at 6 °C (Fletcher 1994), and mesophyll cells have been stored at 4 °C, but for only one month (Jullien 1983).

The present research was carried out to devise a simple and effective method for *in vitro* long-term storage of *Asparagus officinalis* tissue cultures through slow growth techniques.

Materials and methods

Supermale line of *Asparagus officinalis* L. cv. U.C. 157 was used. Young spears were taken and apicals ranging in length from 2 to 3 cm were surface sterilized by 70 % ethanol for 1 min followed by 1.5 % sodium hypochlorite solution for 10 min and then rinsed three times with sterile distilled water. Shoot tips (2 - 3 mm) were excised and cultured in glass tubes (100 × 25 mm) contained 20 cm^3 of Murashige and Skoog (MS) medium supplemented with 1 mg dm^{-3} kinetin (Kin) + 0.5 mg dm^{-3} naphthaleneactic acid (NAA). For shoot multiplication, nodal segments of the proliferated shoots were vertically transferred to jars (100 × 50 mm) containing 40 cm^3 of the same medium. For callus induction, shoot segments (about 1 cm long) were cut and horizontally cultured on

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Abbreviations: BA - benzyladenine; Kin - kinetin; MS - Murashige and Skoog; NAA - naphthaleneactic acid.
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medium contained 1 mg dm^{-3} NAA + 0.5 mg dm^{-3} benzyladenine (BA). The induced callus were recultured twice on fresh medium to obtain stock materials.

The media used contained 30 g dm^{-3} sucrose and 7 g dm^{-3} agar and were adjusted to pH 5.8 before autoclaving. The normal incubation conditions were: temperature of 25 ± 2 °C, photoperiod 16 h, irradiance of 45 µmol m^{-2} s^{-1} (*Philips* white fluorescent tubes). Before placing cultures in the storage conditions, all were incubated for 5 d at 28 °C to allow elimination of contaminated specimens.

For cold storage of shoot cultures, the proliferated shoots (length of 1.5 cm) were individually transferred to fresh multiplication medium (MS + 1 mg dm^{-3} Kin + 0.5 mg dm^{-3} NAA) and then incubated at 5 °C in the dark. Twenty cultures were taken after 1, 6, 12 and 18 months and survival percentage, healthy shoot percentage, number of proliferated shoots and height of shoots were recorded. The cultures were transferred to fresh medium and placed under standard culture room conditions for four weeks and then survival percentage were assessed (survival on recovery conditions).

For cold storage of callus cultures, equal inoculum (about 250 mg) of the proliferated callus were transferred to callus induction medium (MS + 1 mg dm^{-3} NAA + 0.5 mg dm^{-3} BA) and then they were incubated at 5 °C in the dark. Eighty replicates were used in this experiment. After 1, 6, 12 and 18 months, fresh mass, dry mass, dry mass/fresh mass and percentages of embryonic cultures were recorded from twenty replicates of each treatment.

To asses the importance of increased sugar content and osmotic stress in the long-term storage, shoots (1.5 cm) were individually cultured on multiplication medium supplemented with 40 g dm^{-3} mannitol. Survival percentages, healthy shoot percentages, number of proliferated shoots, and height of the shoots were recorded after 1, 6, 12 and 18 months of incubation at normal conditions. Each treatment consists of twenty replicates. The cultures were transferred to mannitol-free medium and survival percentages were recorded after four weeks of transferring.

Pieces (about 250 mg) of callus were subcultured on callus induction medium containing 40 g dm^{-3} mannitol and then the cultures were incubated at normal conditions. Twenty replicates were taken after 1, 6, 12 and 18 months and fresh mass, dry mass, growth value, dry matter content and percentage of embryogenic cultures were assessed.

All experiments were designed in completely randomized design and obtained data were statistically analyzed using standard error (SE) according to the method described by Snedecor and Cochran (1967).

Results and discussion

Low-temperature storage: The shoots stored up to 6 month remained fully viable (100 % survival), and low rate of multiplication and low height of shoots were observed (Table 1, Fig. 1*A*). After an additional 6 months 90 % of shoot were alive, but some shoots became white and succulent (Fig. 1*B*). However, 50 % of shoots remained healthy after 18 months of storage. This percentage was improved when shoots were recultured at normal conditions (Table 1).

The fresh mass of calli increased till twelfth month of the storage. However, dry mass, dry mass/fresh mass and percentage of embryogenic cultures were decreased after 6 months of storage. It is important here to mention that, callus cultures remained healthy without any sign of senescence after 12 months of storage at low temperature conditions (Fig. 2B). These results are in line with those obtained by Fletcher (1994). He mentioned that the cultures of asparagus remained viable after 15 - 16 months of storage at 6 °C. The shoots of *Saussurea lappa* stored *in vitro* at 5 °C in dark up to 12 months remained viable although the medium desiccated considerably (Arora and Bhojwani 1989). Also 85 % of apple shoot

Table 1. Survival and proliferation of *Asparagus officinalis* shoot cultures during storage at low temperature of 5 °C or osmotic stress induced by 40 g dm^{-3} mannitol. Means ± SE, $n = 20$.

Storage duration [month]	Survival [%]		Healthy shoots [%]		Number of proliferated shoots		Height of shoot [cm]		Recovery [%]	
	5 °C	mannitol	5 °C	mannitol	5 °C	mannitol	5 °C	mannitol	5 °C	mannitol
1	100	100	100	100	2.50±0.20	3.40±0.10	1.50±0.09	2.60±0.30	100	100
6	100	100	100	90	3.00±0.08	4.80±0.12	2.60±0.11	4.30±0.33	100	100
12	90	50	85	30	3.30±0.40	4.00±0.20	3.50±0.21	4.50±0.25	90	40
18	60	-	50	-	3.35±0.18	-	4.50±0.20	-	65	-

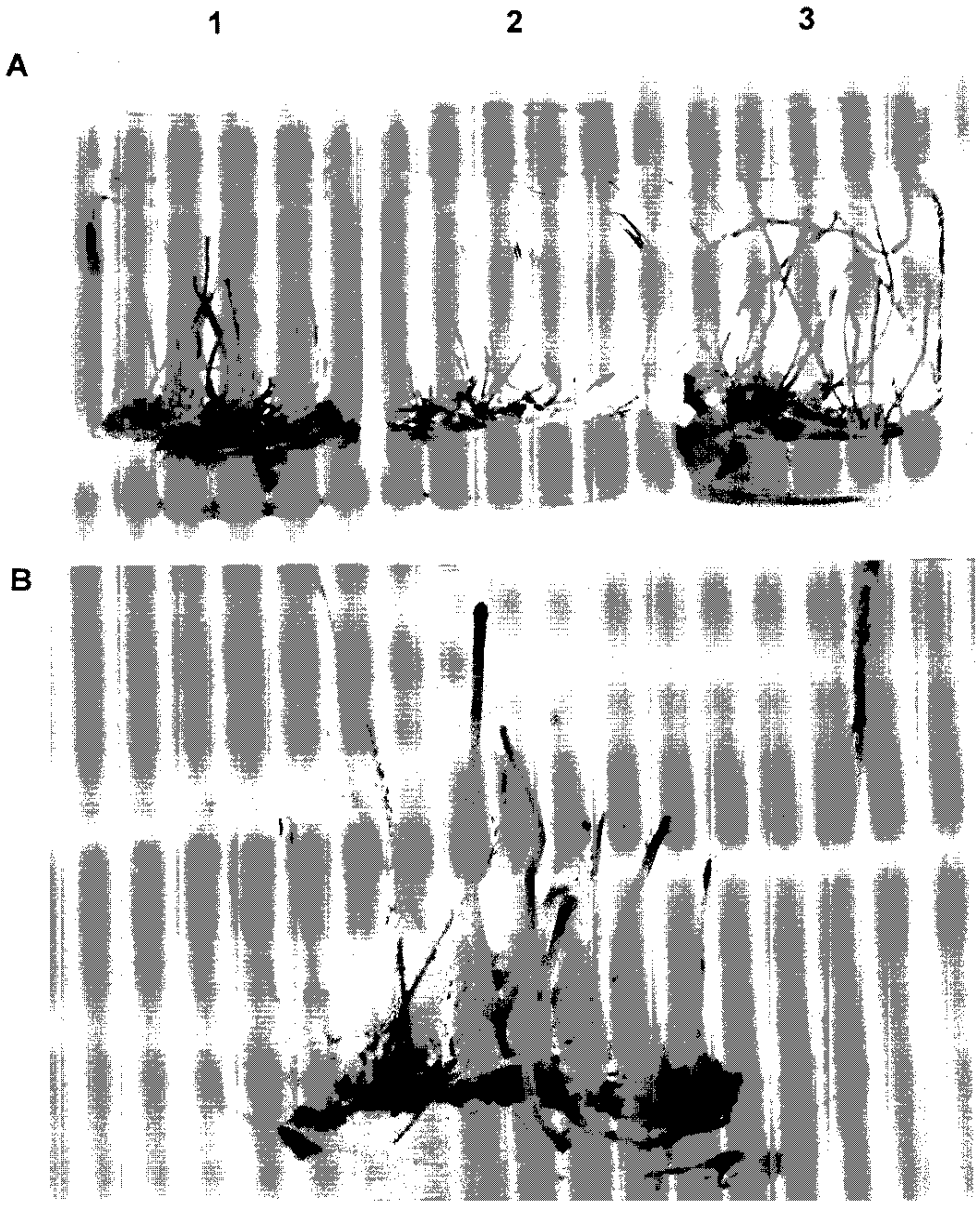

Fig. 1. *Asparagus officinalis* shoot cultures: *A* - after six months of growth on medium contained 40 g dm^{-3} mannitol (*1*), at 5 °C in the dark (*2*), and normal growth conditions (*3*); *B* - after 12 months of growth at 5 °C in the dark.

cultures and 80 % of pear shoot cultures remained alive after 18 months storage at 8 °C and 4 °C, respectively, in the dark (Wanas *et al.* 1986, Wanas 1992). In this respect, Dodds (1988) mentioned that at temperature lower than 3 °C potato *in vitro* cultures experience frost damage, but 6 °C seems to be well tolerated.

Modified medium storage: To slow the growth of asparagus cultures, the supplementation of culture medium by 40 g dm^{-3} mannitol was used, since mannitol is reported to inhibit growth of micropropagated asparagus (Conner and Fallon 1993).

Up to 6 months at normal culture conditions, 90 % of shoot cultures remained healthy and green on medium with 40 g dm^{-3} mannitol. Also normal morphology and low multiplication rate were observed (Table 1, Fig. 1*A*). However, decline in shoot viability was detected after 12 months (50 % survival and 30 % healthy cultures) and the rate of shoot proliferation was further decreased.

Table 2. Growth and differentiation of *Asparagus officinalis* callus cultures during storage at low temperature of 5 °C or osmotic stress induced by 40 g dm^{-3} mannitol. Means ± SE. $n = 20$. Initial fresh mass 250 mg.

Storage [month]	Fresh mass [g]		Dry mass [mg]		Dry mass / fresh mass		Embryogenic calli [%]	
	5 °C	mannitol	5 °C	mannitol	5 °C	mannitol	5 °C	mannitol
1	1.00±0.10	0.90±0.10	105.25±10.00	100.00± 5.00	0.105	0.111	10	20
6	1.12±0.11	1.10±0.20	112.00± 9.00	120.20±11.00	0.100	0.109	20	30
12	1.30±0.09	0.55±0.15	110.00± 8.00	65.00± 5.00	0.084	0.118	10	-
18	1.10±0.10	-	90.50± 8.00	-	0.081	-	-	-

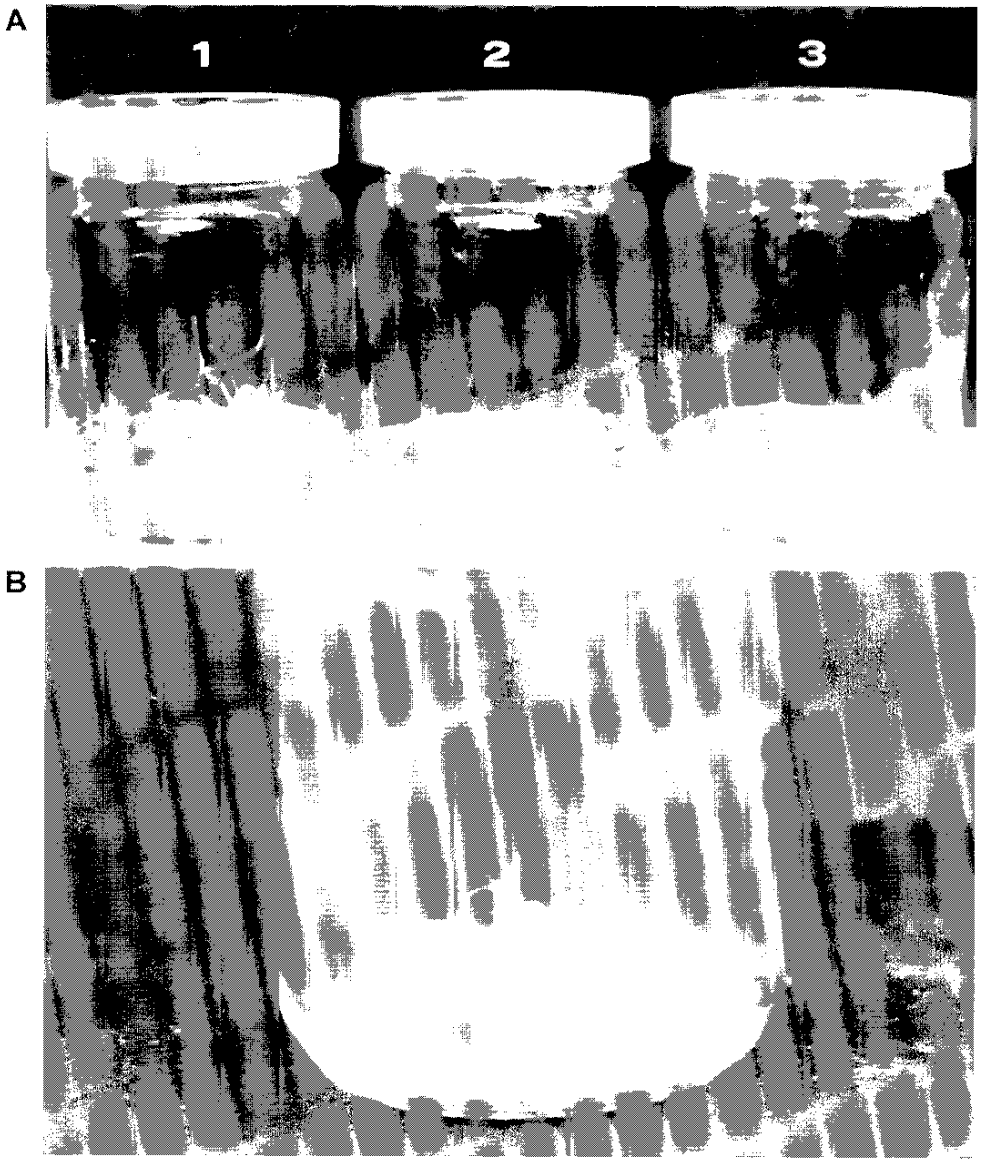

Fig. 2. Callus cultures of *Asparagus officinalis*: *A* - after six months of growth on medium containing 40 g dm^{-3} mannitol (*1*), at 5 °C in the dark (*2*) and normal growth conditions (*3*); *B* - after 12 months of growth at 5 °C in the dark.

Data of callus growth dynamics on medium containing mannitol indicated that fresh mass, dry mass and percentage of embryogenic calli were increased till sixth month (Table 2, Fig. 2*A*). The highest dry and fresh masses were recorded after 12 months of growth, however, a sharp drop in the growth parameters was observed after 12 months of storage. All the callus cultures were dried and died after 18 months of storage.

In this respect, several authors claimed that, high sugar concentrations in culture medium, increased survival of *in vitro* stored cultures, *e.g.* shoot cultures of asparagus survived for 20 months when stored on medium containing 30 % sucrose and 4 % sorbitol (Fletcher 1994). The longevity of cassava tissue cultures stored at -22 °C increased with increased sucrose concentration up to 40 % (Roca *et al.* 1982).

In conclusion, the low temperature storage seems to be most applicable to the *in vitro* germplasm storage of *Asparagus officinalis*. While modified sugar concentrations could be applied in order to expand the duration of reculturing and subsequently to save the expensive subculturing requirements.

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