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ORIGINAL ARTICLE

Cold Storage of Plant Tissue Cultures – Effect of Sucrose

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Explants of normal (non-transformed) carrot (*Daucus carota* L.) tissue, scorzonera crown gall (*Scorzonera hispanica* L.) and carrot tissues transformed with *Agrobacterium tumefaciens* (all cultured *in vitro*) were planted in little glass jars with 6 cm^3 of nutrition media containing 3, 5, 7 or 9% sucrose and maintained at +3 ºC for 6, 8, 10 and 12 months. Viability of tissue cultures after cold storage was estimated by the degree of tissue necrosis, per cent of explants exhibiting growth resumption and intensity of the resumpted growth. Little or no growth was observed during cold storage of scorzonera and normal carrot tissues, whereas transformed carrot tissues showed significant growth. Dry matter content of tissues increased with an increase of sucrose concentration in the medium and failed to change during cold storage of scorzonera and normal carrot tissues. The dry matter content of the transformed carrot tissues fell down during cold storage due to the increase of fresh weight. The viability of all tissue cultures was preserved better and for a longer time with 7% sucrose, than with 3, 5 and 9%. Transformed carrot and tobacco callus tissues preserved cell viability better than non-transformed ones. Cycles consisting from long subcultures (several months) at 3 ºC interrupted by short subcultures (several weeks) at 26 ºC were applied in order to storage of tissue cultures for several years.

Key words: Agrobacterium tumefaciens, carrot, cold storage Daucus carota, scorzonera, Scorzonera hispanica, sucrose, tobacco, transformation

Laboratories working with plant tissue cultures need methods for their prolonged storage which must be low labor-consuming and not expensive. Two methods of plant tissue culture conservation have been now worked out: 1) long-term conservation in liquid nitrogen, 2) medium-term conservation which is achieved due to a great increase of subculture duration by modification of the growth medium, limitation of oxygen access or by cultivation at low temperatures (Withers, 1985; Engelmann, 1996).

Most experimental works on tissue culture storage are devoted to cryoconservation. However, storage of tissue cultures without their freezing in liquid nitrogen has some advantages such as simpler preparation of tissues for storage, possibility for checking viability of tissues during storage, no need of liquid nitrogen and expensive equipment. One of methods for prolonged conservation is the cultivation at low above zero temperatures what gives the possibility to increase subculture period to 12 or even more months (Kovalchuk *et al*., 2009; Romano *et al*., 1999; Luo *et al*., 2008; Gamburg, 2013; Bekhet *et al*., 2016). Luo *et al*. (2008) compared cold storage at 4º and cryopreservation of some hairy-root cultures and went to the conclusion than cold storage is the better way of conservation. «Hairy» roots of ginseng were stored at 4 ºC for 4 months (Yoshimatsu *et al*., 1996). Alginate-encapsulated shoot apexes of some tropical trees showed viability after 12 months at 12 ºC (Maruyama *et al*., 1997). Soybean callus resumed growth after 154 days of storage at 10 ºC (Boysen Kirsten, Wyndaele, 1985). Tissue cultures of *Catharanthus roseus, Voacanda thouarsii and Coffea arabica* were stored at 15 ºC for 4 months (Augereau, 1987). Protocorms of orchids (Dubus, 1980), shoot apexes of *Actinidia deliciosa* (Monette, 1987) and strawberry plantlets (Reed, 1991) were successfully stored at low temperatures for one year. Many authors used for cold storage the same medium as for cultivation at optimal temperatures.

Some modifications of the medium composition are assumed to improve the viability preservation during cold storage of plant tissue cultures. In this article we reported on the influence of different sucrose concentrations in the medium on the prolonged preservation of scorzonera, carrot and tobacco tissues cultures at 3 ºC.

MATERIALS AND METHODS

Tissue cultures. The list of tissue cultures used in this work is presented in Table 1. Normal carrot (*Daucus carota* L., cv. Nantskaya 4) tissue culture was obtained from explants of storage roots cultured on the medium containing MS salts (Murashige & Skoog, 1962), 3% sucrose, thiamin 1 mg l^{-1}, *myo*-inositol 80 mg l^{-1}, 2,4-D 0.03 mg l^{-1}, kinetin 1 mg l^{-1}, agar 7-8 g l^{-1}, pH 5.6 (Markova *et al*., 1995). The callus appeared was subcultured on the same medium every 28 days. Transformed carrot tissue cultures were obtained by inoculation of the surface of storage root discs with tumor-inducing *Agrobacterium tumefaciens* strains 8628 and C58C1. The discs were incubated for 3 days on the medium mentioned above lacking sucrose and growth regulators and then transferred to the medium with 3% sucrose, 500 mg l^{-1} carbenicillin and lacking 2,4-D and kinetin. Crown gall tissues thus obtained were subcultured two times on the medium with carbenicillin for elimination of bacteria and then subcultured every 28 days on the medium for normal carrot tissue lacking growth regulators. These tissue cultures showed no demand for auxin and cytokinin for their growth; they synthesized octopine and possessed the indoleacetamido-hydrolase activity (Markova *et al*., 1995). It was assumed that these tissues were transformed.

Tissue culture of scorzonera crown gall tumor was received in 1972 from the Institute of Experimental Botany (Prague, Czechia) and maintained up to now in tissue and cell cultures. The strain of *A. tumefaciens* used for tumor induction is unknown, but it was shown that the tissue contained octopine and possessed indoleacetamido-hydrolase activity (Markova *et al*., 1995) what conformed that this tissue culture is transformed. The tissue culture of scorzonera was subcultured every 21 days on the medium with salts of the B5 medium (Gamborg *et al*., 1968), 3% sucrose, thiamine 0.4 mg l^{-1}, pyridoxine 0.1mg l^{-1} and agar 7-8 g l^{-1}, pH 5.6. All tissue cultures were maintained in darkness at 26 º C.

Tobacco (*Nicotiana tabacum* L.) tissue cultures were obtained from leaf mesophyll of wild and transformed with disarmed strain of *Agrobacterium tumefaciens* A699 plants. Transformed nature of calli and regenerated plants was conformed by insensitivity to kanamycin 50 mg/l and by the PCR with primer to *nptII* gene. Tissue culture were grown on the medium containing MS salts, thiamine 1 mg l^{-1}, pyridoxine 0.5 mg l^{-1}, nicotinic acid 0.5 mg l^{-1}, *myo*-inositol 80 mg l^{-1}, sucrose 3%, NAA 1 mg l^{-1}, agar 0.8%. Subculture duration was 30 days. All tissue cultures were maintained in darkness at 26 ºC.

Experimental procedure. Explants of tissue cultures (50-70 mg fr. wt.) at stationary growth phase were planted in glass jars (76 mm height and 26 mm diameter) with 6 cm^3 of media containing 3, 5, 7 or 9% sucrose. The jars were tightly covered with aluminum foil and incubated for 6 days at 26 º C, then transferred to a thermostat at 3 º C and stored in darkness for 4, 6, 8, 10 and 12 months. Fresh and dry weights of the explants and their viability were determined at times indicated. Tissue viability was estimated by a degree of necrosis and the ability for growth resumption. For this purpose, the jars with scorzonera tissue were transferred to 26 ºC for 14 days and a number of growing explants was determined. Grown tissue was planted on the medium with 3% sucrose, cultivated for 21 days at 26 ºC and the growth index was calculated as a final to initial fresh weights ratio. Carrot tissues were transferred after cold storage to 26 ºC for one day. The degree of tissue necrosis was visually estimated and some parts of remaining alive tissues were cultured for 28 days on the medium with 3% sucrose at 26 ºC. The growth index and number of growing explants were calculated at the end of the subculture. Growth of tissue cultures not undergone to cold storage was also determined on media with different sucrose concentrations.

Statistical analysis. Each experimental variant had 15 jars, 5 of them were used for determination of fresh and dry weights at the end of cold storage and 10 ones for the analysis of growth restoration. Mean values and their statistical errors are presented in tables.

RESULTS

It is desirable to use small cultural flasks with little medium volume and small explants for cold storage of tissue cultures in order to reduce the requirement of space in low-temperature thermostat. The jars with tissue explants used in this work were small enough for this purpose. They were tightly closed with aluminum foil in order to prevent contamination and loss of water during long storage (up to one year).

As shown in Table 2, all tissue cultures grew well at control conditions (3% sucrose and 26°) in spite of these limitations. An increase of sucrose concentration from 3 to 5% caused growth stimulation of all tissue cultures estimated by fresh and dry weights. More high sucrose concentrations (7 and 9%) diminished final fresh weight in comparison with 3 and 5%, but final dry weight

was not diminished at 7% sucrose concentration. All transformed tissue cultures showed faster growth than normal carrot tissue culture. Growth of transformed carrot tissue cultures proceeded especially intensively. As a result, fresh weight of tissues achieved 47 and 68% of fresh weight of media (approximately 6 g) for tissue cultures transformed with *A. tumefaciens* strains 8628 and C58C1 consequently. The per cent of tissue dry weights at the end of subculture increased significantly according to the increase of sucrose concentrations. It may be concluded that tissue cultures used in this work could grow in small flasks.

Fresh weights of tissue explants increased 1.5-3 times and dry matter contents 1.8-2.5 times during preliminary (before cold storage) 6 day incubation at 26 ºC depending on tissue culture and sucrose concentration (data not shown). This preliminary cultivation was necessary for cell restoration after starvation during stationary phase of the preceding cultural cycle. Further changes of the fresh and dry weights of explants during incubation at 3 ºC are presented in Table 3. Fresh and dry weights of scorzonera tissue explants showed little changes between 6 and 10 months of cold storage and fresh weight was smaller at higher sucrose concentrations. Scorzonera tissue culture remained alive at all sucrose concentrations for 10 months, but became dyed to 12th month of cold storage. There were no significant differences in tissues fresh and dry weights between sucrose concentrations and duration of cold storage from 6 to 10 months. However, growth was severely inhibited at 9% sucrose.

The growth of non-transformed carrot tissue culture at 3% sucrose continued for 8 months and then stopped and tissues became dyed to 12 months. There were no significant increase of fresh weight from 4th to 8th months of cultivation, but some burst of it proceeded in last 4 months. 9% sucrose was inhibitory in comparison with its lower concentrations. Slow growth of the fresh and dry weights of normal carrot tissue explants was observed at the period from 4 to 12 months of cold storage especially at 7% sucrose.

Fresh and dry weight of the explants of transformed carrot tissues increased significantly during first 4 months of cold storage. Fresh weight continued to grow up to 8 month at 5 and 7% sucrose and decreased after 4-6 months at 3% sucrose and after 8 months at 5% sucrose. Dry weight of the explants did not change significantly during 4-12 months of cold storage.

Dry matter content of scorzonera tissue explants after 6 months of cold storage (Table 4) was higher than at the end of the subculture at 26 ºC (Table 1) and showed no (3% sucrose) or little (5-7% sucrose) increase during subsequent 4 months of storage. The dry matter content of normal carrot tissue during 4-12 months of cold storage was very high (15-18%) when compared to other tissue cultures (Table 4). It was somewhat higher at 5 and 7% sucrose than at 3% sucrose after 4 months of storage and this difference decreased and disappeared during subsequent months. The dry matter content of transformed carrot tissues was 7-9% after 4 months of cold storage and fell down at the 8th month when fresh weight of explants was maximum.

Necrotic tissue of the normal and transformed carrot tissue cultures had brown color and living tissue was yellowish white. Thus, a degree of tissue necrosis could be readily estimated. Necrosis of normal carrot tissue after 6 months of cold storage amounted to 94-98% of total tissue volume at 3 and 5% sucrose, whereas only 42% of tissue was necrotic at 7% sucrose (Table 5). Necrosis of almost all tissue was observed after 8-

12 months of cold storage independently of sucrose concentration. In spite of this, it was possible to find pieces of living tissue to use them for the following subculture at 26 ºC. Transformed carrot tissues showed no signs of necrosis after 6 and even 8 (C58C1) months of cold storage. Necrosis increased to 75-100% during further storage and this necrotization proceeded more slowly at 7% sucrose than at 3 and 5%.

Table 1. Tissue cultures used in the work.

Tissue cultures	**Growth regulators in media**
Crown-gall scorzonera tissue culture	No
Carrot tissue culture	2,4-D 0.03 mg l^{-1}, kinetin 1 mg l^{-1}
Carrot tissue culture transformed with *A. t.* (8628)	No
Carrot tissue culture transformed with *A. t.* (C58C1)	No
Tobacco tissue culture	NAA 1 mg l^{-1}
Tobacco tissue culture transformed with *A. t.* (699)	NAA 1 mg l^{-1}

Table 2. Fresh and dry weights, growth index (final : initial fresh weight) and dry matter content of tissues at the end of subculture at 26 ºC (21 days for scorzonera and 28 days for carrot tissue cultures) on media with different sucrose concentrations.

Sucrose, %	**Fresh weight, g**	**Growth index**	**Dry weight, mg**	**Dry matter content, %**
Scorzonera tissue culture				
3	1.78 ± 0.09	22.0 ± 1.1	62 ± 7	3.48 ± 0.39
5	1.94 ± 0.07	24.0 ± 0.8	113 ± 4	5.80 ± 0.10
7	1.27 ± 0.25	15.7 ± 3.1	104 ± 20	8.20 ± 0.24
9	0.77 ± 0.08	9.5 ± 1.0	86 ± 7	11.46 ± 0.88
Normal carrot tissue culture				
3	0.85 ± 0.06	10.8 ± 0.8	51 ± 4	6.04 ± 0.09
5	1.33 ± 0.15	17.0 ± 1.9	97 ± 10	7.30 ± 0.12
7	1.15 ± 0.08	14.8 ± 1.0	108 ± 2	9.51 ± 0.60
9	0.59 ± 0.10	7.6 ± 1.3	76 ± 12	12.93 ± 0.21
Transformed (8628) carrot tissue culture				
3	2.03 ± 0.38	39.8 ± 7.5	62 ± 8	3.16 ± 0.18
5	2.80 ± 0.30	54.9 ± 5.9	126 ± 10	4.59 ± 0.26
7	1.53 ± 0.15	30.1 ± 2.9	117 ± 10	7.63 ± 0.10
9	0.64 ± 0.11	12.6 ± 2.2	67 ± 12	10.40 ± 0.11
Transformed (C58C1) carrot tissue culture				
3	2.58 ± 0.34	36.4 ± 4.8	85 ± 5	3.48 ± 0.34
5	3.83 ± 0.10	53.9 ± 1.4	147 ± 14	3.87 ± 0.43
7	2.09 ± 0.64	29.5 ± 9.1	156 ± 32	8.05 ± 0.69
9	1.24 ± 0.23	17.5 ± 3.2	143 ± 21	11.75 ± 0.34

Table 3. Changes of fresh weight (FW, g) and dry weight (DW, mg) of tissues during storage at 3 ºC with different sucrose concentrations.

Sucrose, %	Duration of storage, months				
	4	6	8	10	12
Scorzonera tissue culture					
3 FW	-	0.28 ± 0.07	0.44 ± 0.09	0.44 ± 0.03	-
DW	-	21 ± 5	39 ± 10	32 ± 2	-
5 FW	-	0.29 ± 0.05	0.32 ± 0.06	0.30 ± 0.06	-
DW	-	26 ± 4	28 ± 5	32 ± 6	-
7 FW	-	0.24 ± 0.08	0.23 ± 0.04	0.19 ± 0.04	-
DW	-	26 ± 9	30 ± 9	24 ± 9	-
9 FW	-	-	-	0.12 ± 0.02	-
DW	-	-	-	19 ± 4	-
Normal carrot tissue culture					
3 FW	0.24 ± 0.05	0.32 ± 0.08	0.42 ± 0.04	0.33 ± 0.06	-
DW	56 ± 6	40 ± 9	44 ± 4	57 ± 17	-
5 FW	0.38 ± 0.06	0.43 ± 0.12	0.34 ± 0.09	0.50 ± 0.06	0.55 ± 0.10
DW	75 ± 22	58 ± 12	55 ± 13	65 ± 4	72 ± 7
7 FW	0.27 ± 0.08	0.33 ± 0.03	0.32 ± 0.07	0.61 ± 0.14	0.66 ± 0.11
DW	48 ± 14	54 ± 12	50 ± 9	81 ± 15	99 ± 14
9 FW	-	-	-	0.29 ± 0.08	0.44 ± 0.06
DW	-	-	-	40 ± 6	84 ± 10
Transformed (8628) carrot tissue culture					
3 FW	0.94 ± 0.15	2.01 ± 0.08	1.30 ± 0.18	1.09 ± 0.10	-
DW	66 ± 6	61 ± 2	50 ± 2	49 ± 1	-
5 FW	1.90 ± 0.32	2.58 ± 0.19	3.14 ± 0.29	2.02 ± 0.19	2.58 ± 0.40
DW	122 ± 10	126 ± 6	100 ± 6	88 ± 6	151 ± 68
7 FW	2.08 ± 0.44	1.47 ± 0.15	3.16 ± 0.32	2.66 ± 0.36	2.85 ± 0.21
DW	168 ± 25	150 ± 9	159 ± 10	138 ± 5	135 ± 3
9 FW	-	-	-	2.21 ± 0.49	1.66 ± 0.49
DW	-	-	-	155 ± 12	153 ± 14
Transformed (C58C1) carrot tissue culture					
3 FW	1.78 ± 0.07	1.20 ± 0.13	1.40 ± 0.10	1.15 ± 0.09	-
DW	76 ± 0	64 ± 3	58 ± 2	52 ± 1	-
5 FW	0.66 ± 0.13	0.90 ± 0.13	2.87 ± 0.18	2.10 ± 0.29	1.88 ± 0.08
DW	61 ± 10	90 ± 8	111 ± 1	127 ± 24	96 ± 5
7 FW	1.34 ± 0.17	1.59 ± 0.15	2.13 ± 0.32	2.24 ± 0.18	2.22 ± 0.28
DW	127 ± 13	151 ± 16	161 ± 12	161 ± 6	146 ± 8
9 FW	-	-	-	1.40 ± 0.28	3.41 ± 0.29
DW	-	-	-	185 ± 28	243 ± 15

Table 4. Dry matter content (%) of tissues cultured at 3 º C with different sucrose concentrations in media.

Sucrose,	Duration of cold storage, months				
%	4	6	8	10	12
Scorzonera tissue culture					
3	-	7.4 ± 0.2	9.1 ± 1.6	7.4 ± 0.2	-
5	-	9.0 ± 0.3	9.0 ± 0.2	10.9 ± 0.5	-
7	-	11.0 ± 0.1	12.4 ± 1.4	12.9 ± 0.3	-
9	-	-	-	15.6 ± 0.4	-
Normal carrot tissue culture					
3	14.7 ± 0.7	13.2 ± 0.5	11.5 ± 1.4	13.5 ± 1.5	-
5	19.0 ± 3.8	14,5 ± 1.0	16.7 ± 0.6	13.4 ± 0.7	13.9 ± 1.4
7	18.0 ± 0.5	17.6 ± 0.9	16.6 ± 0.8	14.4 ± 1.4	15.3 ± 0.8
9	-	-	-	18.5 ± 1.6	19.0 ± 0.3
Transformed (8628) carrot tissue culture					
3	7.3 ± 0.5	3.1 ± 0.2	4.2 ± 0.7	4.6 ± 0.4	-
5	7.0 ± 0.8	5.0 ± 0.3	3.3 ± 0.3	3.8 ± 0.5	5.6 ± 1.8
7	8.8 ± 0.9	10.4 ± 0.7	5.2 ± 0.7	5.7 ± 1.0	4.9 ± 0.6
9	-	-	-	8.3 ± 1.4	11.0 ± 1.6
Transformed (C58C1) carrot tissue culture					
3	4.3 ± 0.2	5.6 ± 0.6	4.2 ± 0.4	4.6 ± 0.4	-
5	9.6 ± 0.6	9.3 ± 0.7	4.5 ± 0.4	5.3 ± 0.8	5.2 ± 0.5
7	9.6 ± 0.5	9.5 ± 0.5	8.0 ± 0.7	7.4 ± 0.8	6.9 ± 0.7
9	-	-	-	13.7 ± 0.9	7.3 ± 0.5

Table 5. Degree of necrosis (%) of carrot tissues cultured at 3 ºC with different sucrose concentrations in media.

Sucrose,	Duration of cold storage, months			
%	6	8	10	12
Normal tissue culture				
3	98 ± 2	98 ± 2	99 ± 1	-
5	94 ± 3	97 ± 2	97 ± 2	97 ± 2
7	42 ± 7	94 ± 3	96 ± 2	97 ± 2
9	-	-	89 ± 3	96 ± 2
Transformed (8628) tissue culture				
3	0	70 ± 6	78 ± 7	-
5	0	89 ± 7	92 ± 5	100 ± 0
7	0	51 ± 6	75 ± 10	97 ± 2
9	-	-	80 ± 9	96 ± 3
Transformed (C58C1) tissue culture				
3	0	0	78 ± 7	-
5	0	0	91 ± 5	87 ± 5
7	0	0	75 ± 10	92 ± 5
9	-	-	80 ± 9	84 ± 7

Table 6. Growth resumption of tissues cultured after cold storage at 3 ºC with different sucrose concentrations.

Sucrose,	Duration of cold storage, months							
%	6		8		10		12	
	n*	GI**	n	GI	n	GI	n	GI
Scorzonera tissue culture								
3	7	28.7 ± 3.5	1	31.6	2	6.7 ± 0.6	-	-
5	9	41.6 ± 3.4	6	29.5 ± 3.8	1	7.4	-	-
7	10	28.7 ± 3.1	8	25.4 ± 2.6	5	11.9 ± 2.6	-	-
Normal carrot tissue culture								
3	1	1.6	-	-	1	2.8	-	-
5	3	11.3 ± 2.5	-	-	2	4.3 ± 0.1	1	11.6
7	7	8.4 ± 1.4	-	-	4	9.3 ± 1.4	2	14.7 ± 0.1
9	-		-	-	7	10.5 ± 1.8	3	4.8 ± 1.4
Transformed (8628) carrot tissue culture								
3	6	3.2 ± 0.4	2	6.4 ± 3.5	0	0	-	-
5	10	6.3 ± 1.0	9	19.2 ± 4.7	3	9.0 ± 1.8	0	0
7	10	8.9 ± 1.9	10	25.3 ± 3.1	6	21.7 ± 0.6	2	20.2 ± 4.3
9	-	-	-	-	5	18.3 ± 3.5	2	30.1 ± 2.1
Transformed (C58C1) carrot tissue culture								
3	7	7.5 ± 2.0	4	5.4 ± 2.4	2	6.1 ± 1.0	-	-
5	10	20.6 ± 2.7	9	15.4 ± 1.5	6	26.1 ± 4.5	5	17.8 ± 4.0
7	10	20.5 ± 2.6	3	19.3 ± 0.7	7	28.6 ± 2.3	3	21.7 ± 7.4
9	-	-	-	-	6	25.2 ± 4.7	5	29.6 ± 2.4

* number of growing explants (total number of explants = 10)

** growth index (fresh weight final/initial)

Table 7. Growth index of normal (NtN) and transformed (Nt699) tobacco tissue cultures after their prolonged storage at 3° C and 7% sucrose in the medium.

Months	1st experiment		2nd experiment	
	NtN	Nt699	NtN	Nt699
4	8,3 ± 2,0	17,8 ± 3,1	3,6 ± 1,0	18,2 ± 3,1
5	1,1 ± 0,1	11,9 ± 0,2	1,6 ± 0,2	6,5 ± 1,9
6	1,5 ± 0,2	4,1 ± 0,8	2,0 ± 0,4	5,9 ± 0,9

The data on growth restoration after cold storage are presented in Table 6. 70% of scorzonera tissue explants stored for 6 months at 3% sucrose grew at 26 ºC. Prolongation of cold storage to 8 and 10 months resulted in a sharp decrease of the number of explants capable of growth resumption. Almost all explants resumed growth after 6 months of cold storage at 5 and 7% sucrose and their number decreased during following storage. However, even after 10 months half of the explants stored at 7% sucrose were capable to grow. The intensity of growth resumption after 6 and 8 months of cold storage (estimated as growth index) remained comparable

to that of tissue which did not undergo cold storage (see Table 2) independently of sucrose concentration. A decline of growth index was observed after 10 months of cold storage. Normal carrot tissue could not be stored at 3% sucrose because only one of ten explants showed very weak growth after 6 and 10 months of cold storage (Table 6). An increase of sucrose concentration caused better growth resumption after cold storage, and the best results were observed at 7 and 9% sucrose. Transformed carrot tissues showed better viability preservation than the normal one. More than half of explants resumed growth after 10 months of cold storage at 7% sucrose and the growth rate of these explants was comparable to that of explants not stored at 3 ºC (see Table 2). As shown in Table 7, transformed tobacco tissue was also able to grow faster than normal one after 6 months of cold storage with the presence of 7% sucrose in the medium.

DISCUSSION

As shown in our experiments, preservation of tissue cultures at low temperature may be significantly improved due to the increase of sucrose concentration in the medium. An optimum level of sucrose was shown to be 7% for all our tissue cultures. It was impossible to increase viability preservation by further increase of sucrose concentration. An increase of tolerance to cryopreservation was achieved by growing tissue cultures in sucrose-enriched medium prior to their freezing (Bachiri *et al*., 1995; Thierry *et al*., 1997). Enhanced tolerance to low temperatures of eucalyptus cell suspension culture (Travert *et al*., 1997) and rooted cuttings of garden chrysanthemum (Rajakse *et al*., 1996) was correlated with higher sugar content in cells and leaves. However, these protective effects of sugars were observed in the experiments lasting several days or 4-6 weeks. Our experiments were the first to show the improvement of viability preservation by additional sucrose during 8-10 months of cold storage of plant tissue cultures. The reason for such effect of sucrose is unclear yet. It can be as a result of intracellular osmotic adjustment, defense of biocolloids from dehydration or better and longer provision of cells with respiratory metabolites. A decline of the viability during cold storage from the 6^{th} to the 12^{th} month (Table 5) did not correlate with the changes of dry matter content (Table 2). Therefore it may be assumed that intracellular osmotic conditions have only remote relation to the viability preservation. This assumption can be also deduced from the comparison of normal and tumorous carrot tissue cultures: tumorous tissue cultures preserved the viability better than the normal one, but had significantly lower dry matter content. However, an improving of the viability preservation and an increase of dry matter content because of a rise of sucrose concentration in the medium showed a distinct correlation.

Tumorous carrot tissues were more tolerant to prolonged cold treatment than the normal one (Tables 4 and 5). It may be supposed that this difference is related to the ability of transformed carrot tissues to synthesize phytohormons necessary for their growth. But tobacco tissue culture transformed with disarmed strain of *Agrobacterium tumefaciens* (699) was also more tolerant to cold storage than untransformed one (Table 7). It is probable that some other components of T-DNA entering into transformed cells are responsible for greater resistance of them to cold storage. However, these data are not sufficient for a general conclusion that tumorous transformation of plant cells resulted in an increase of their cold resistance. Greater number of normal and tumorous tissue cultures should be compared in order to conform or to decline this conclusion.

Tumorous carrot tissues grew at 3 ºC so

intensively that to the 8th month of cold storage their fresh weights (near to 3 g per jar) amounted to a half of the initial fresh weight of the medium in the jar (6ml = 6 g). A decline in the viability during subsequent period of cold storage is assumed to be caused not only by cold but also by insufficient supply of nutrients and water. Therefore, a reduction in the growth rate of these tissues at 3 ºC is necessary in order to improve their storage.

It is necessary to search other possibilities for the improving viability preservation and for prolongation of a cold storage period. They can involve the use of growth inhibitors such as abscisic acid (Bornman & Janssen, 1980) and retardants (Sarkar *et al*, 2001), proline (Leddet & Schaeverbeke, 1975) and reducing agents, the changes of mineral salt composition and growth regulator concentration in the medium, regulation of gaseous composition etc.

The temperature of 3 ºC was used in our experiments for cold storage of tissue cultures of cold-tolerant species (carrot, scorzonera, tobacco). Soybean callus was stored for 154 days at 10 ºC (Boysen Kirsten & Wyndaele, 1985) and tissue cultures of *Catharanthus roseus, Voacanda thouarsii* and *Coffea arabica* were stored at 15 ºC for 4 months (Augereau, 1987). Thus, the temperature near or above 10 º C is necessary for cold storage of tissue cultures of cold-sensitive plant species. At least two thermostats are necessary to have in a laboratory for cold storage of plant tissue cultures, one for cold-tolerant species and other for cold-sensitive species.

As a result of our experiments, the cycles of prolonged storage of tissue cultures were developed. At first, tissue cultures grown at 26 º C and 2-3% sucrose were planted in little jars with the medium containing 7% sucrose. They were cultivated for 6 days at 26 ºC and then transferred to the thermostat with 3 º C and stored for 8-10 months. The jars with scorzonera tissue culture were exposed to 26 º C for two weeks after the end of cold storage and then the explants of growing tissue were transferred to the medium with 3% sucrose and cultured as usually at 26 ºC for 21 days. Grown tissue was used in experiments, for suspension culture and for the repeat of cold storage cycle. The jars with carrot tissue cultures were exposed to 26 ºC for one day after cold storage and then pieces of living tissue were planted on the medium with 3% sucrose and cultured at 26 ºC for 28 days. Tissues were used for the experiments and for the next period of cold storage after second subculture at 26 ºC. These cycles of cold storage are used for several years without any aggravation of growth parameters. It was shown also that scorzonera tissue culture retained its ability to synthesize syringaresinol after such storage (Enikeev *et al*., 2001).

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