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EFFECTS OF FREEZING IN LIQUID NITROGEN ON THE PROPERTIES OF A SOYBEAN (*Glycine max* L. var. acme) CALLUS STRAIN USED AS A BIOASSAY FOR CYTOKININ ACTIVITY

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SUMMARY: A soybean callus strain could withstand cryopreservation after a 14 to 48 hours pregrowth with 1 to 1.5 M sucrose followed by slow freezing at 0.5°C.min^{-1} to -40°C before immersion in liquid nitrogen. Cryopreservation did not modify the growth rate of the strain on media containing various concentrations of kinetin and 2-isopentenyl-adenin.

KEY-WORDS: soybean (*Glycine max* L.), callus strain, cryopreservation, cytokinin, bioassay.

INTRODUCTION

Many cell and callus strains are cultivated due to their properties of synthesizing particular compounds. Their propagation in optimal conditions requires regular subcultures, and the strains are not protected from contaminations. Moreover plant cell cultures growing in an undifferentiated state are genetically unstable. The only current technique allowing for the long-term storage of these strains is cryopreservation, i.e. storage in liquid nitrogen (-196°C). However, only a limited number of studies have been carried out concerning the possible modifications of the cell line characteristics induced by cryopreservation (1). Dougall and Whitten (2) were the first authors to show with carrot cells producing anthocyanins that plant cell lines retained their ability to produce specific chemicals after cryopreservation. Similar observations were made notably by Seitz *et al.* (3) for *Digitalis lanata*, Butenko *et al.* (4) for *Dioscorea deltoidea* and *Panax ginseng*, Seitz and Reinhard (5) also for *Panax ginseng*.

A soybean callus strain whose growth rate is cytokinin-dependent is cultivated in our laboratory. It is used as a bioassay for the specific measurement of cytokinic activity in plant extracts added to the culture medium (6, 7). Cryopreservation of soybean has already been carried out using cell suspensions (8, 9) but no technique has been published yet concerning the cryopreservation of this species in the form of callus. In this paper, we present the results concerning the setting up of a cryopreservation method for soybean callus and the effects of deep freezing on its sensitivity to cytokinins.

MATERIALS AND METHODS

In vitro culture

The callus strain utilized originated from cotyledons of *in vitro* germinated plantlets of *Glycine max* L. var. acme. It was cultivated in the dark at 20 ±1°C, on the medium described by Miller (10), in test tubes containing 20 ml of semi-solid medium. Subcultures were carried out every 4 weeks.

Cryopreservation

For cryopreservation experiments, small fragments (around 20 mg fresh weight) were taken from the soft, growing parts of the calluses. For pregrowth, 50 fragments were suspended in 20 ml liquid medium supplemented with sorbitol, sucrose or dimethylsulfoxide at various concentrations, in 100 ml Erlenmeyer flasks, which were placed on a rotary shaker for various durations (14 to 48 hours). Ten pieces of callus were then transferred in each 2 ml cryotube containing 1 ml of the same cryoprotective medium and held for 1 hour at 0°C. The cryotubes were placed in the freezing chamber of a programmable freezing apparatus (Minicool, by L'Air Liquide) and progressively cooled at $0.5°C.min^{-1}$ from 0 to -40°C. Crystallization was carried out at a temperature intermediate between the crystallization and the nucleation temperature of each cryoprotective mixture, by pinching briefly the cryotubes with forceps previously cooled in liquid nitrogen. The cryovials were then immersed in liquid nitrogen and held at -196°C for 24 hours. For thawing, the cryotubes were plunged in a 40°C water bath until the ice melted. The contents of the cryotubes was poured on filter papers placed in 55 mm Petri dishes containing 10 ml semi-solid medium, so as to progressively eliminate the cryoprotective solution (11). After 24 hours, the filter papers with the fragments of callus were transferred onto new Petri dishes. Growth recovery was measured by wheiging regularly the filter papers with the calluses, according to the method developed by Horsch *et al.* (12). Controls corresponded to calluses transferred directly to standard medium without any treatment. The results correspond to the mean value of the measurements made on three independent samples.

Cytokinin bioassay

For the measurement of sensitivity to cytokinins, pieces of calluses (500 to 1000 mg) coming from controls and cultures pregrown for 24 hours with 1 to 1.5 M sucrose and cryopreserved were placed on media containing 10^{-8}, 10^{-6} and 10^{-4} M kinetin or 2-isopentenyl adenin (2 i-P). Their weight increase, expressed in % of their initial fresh weight, was measured after 3 weeks of culture. The results correspond to the mean value of the measurements made on six calluses per condition.

RESULTS

Preliminary experiments with various concentrations of dimethylsulfoxide, sorbitol and sucrose showed the necessity of using sucrose during pregrowth (data not shown). Thus, the effect of 4 sucrose concentrations (0.75, 1, 1.25 and 1.5 M) and 3 pregrowth durations (14 hours, Fig. 1; 24 hours, Fig. 2 ; 48 hours, Fig. 3) was observed. Growth recovery of the controls was immediate, without any lag phase. On the contrary, for cryopreserved calluses, a lag phase of 11 days minimum was observed. This lag phase increased with the duration of pregrowth. No survival was observed for a pregrowth with 0.75 M sucrose. The best

recovery was observed with 1.25 M sucrose for all pregrowth durations. With 1 M, recovery was the lowest for 14 hours, and increased progressively with the pregrowth duration. It was equivalent to that measured with 1 M, for a 24- and 48-hour pretreatment. On the contrary, with 1.5 M sugar, recovery was equivalent to that measured with 1 M for 14 hours, and decreased progressively with increasing pregrowth durations.

The sensitivity to cytokinins (kinetin and 2-iP) was tested with control calluses and cultures frozen after a 24 hours of pregrowth with 1, 1.25 and 1.5 M sucrose. With both hormones, growth rate increased along with higher cytokinin concentrations. No difference was noted between control and cryopreserved calluses.

DISCUSSION/CONCLUSION

This work led to the develpoment of an efficient cryopreservation method for soybean calluses. The optimal conditions set up with soybean calluses differed from that defined for cell suspensions of the same species. Weber *et al.* (9) used increased sorbitol concentrations during pregrowth. Bajaj (8) used 5% dimethylsulfoxide as cryoprotectant and a freezing rate of $2°C.min^{-1}$ down to -100°C. With soybean calluses, the optimal conditions were 1.25 M sucrose and freezing at $0.5°C.min^{-1}$ down to -40°C before immersion in liquid nitrogen. The respective effects of two pregrowth parameters, i.e. duration and sucrose concentration, could be emphasized: with 1 M sugar only, the efficiency of pregrowth increased with its duration. With 1.5 M, cryoprotection was optimal for the shortest pretreatment duration and decreased progressively, thus revealing toxicity linked with increased pregrowth durations with high sucrose concentration. It was also demonstrated that, as previously observed with various cell suspensions (2, 3, 4, 5), cryopreservation had no effect on the properties of this callus line, i.e. its sensitivity to the cytokinin concentration in the culture medium. Therefore, cryopreservation in liquid nitrogen can be used for the safe long-term storage of this soybean callus strain.

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Table 1: Fresh weight increase (in % of the initial fresh weight, ±S.D.) of control and calluses cryopreserved using three different pregrowth treatments (1, 1.25 and1.5M sucrose + LN) after 3 weeks of culture on media containing various concentrations of 2-isopentenyladenin (2-iP) and kinetin (KIN).

	2-iP (M)			KIN (M)		
	10^{-4}	10^{-6}	10^{-8}	10^{-4}	10^{-6}	10^{-8}
1 M + LN	400±21	182±59	116±9	306±78	182±59	119±9
1.25 M + LN	450±49	202±27	146±40	226±42	202±27	132±39
1.5 M + LN	369±48	197±43	122±37	230±19	197±42	122±37
Control	484±66	150±17	126±14	279±74	150±17	126±14

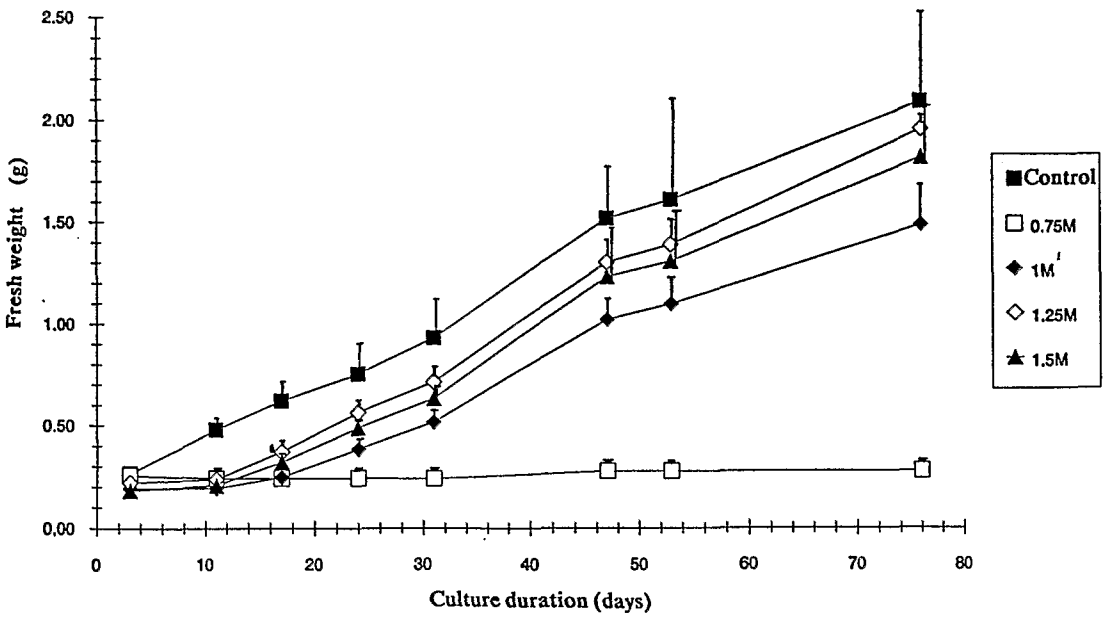

Fig.1: Fresh weight increase of untreated control and cryopreserved soybean calluses, after a 14-hour pregrowth duration with various sucrose concentrations. Vertical bars represent the standard deviation.

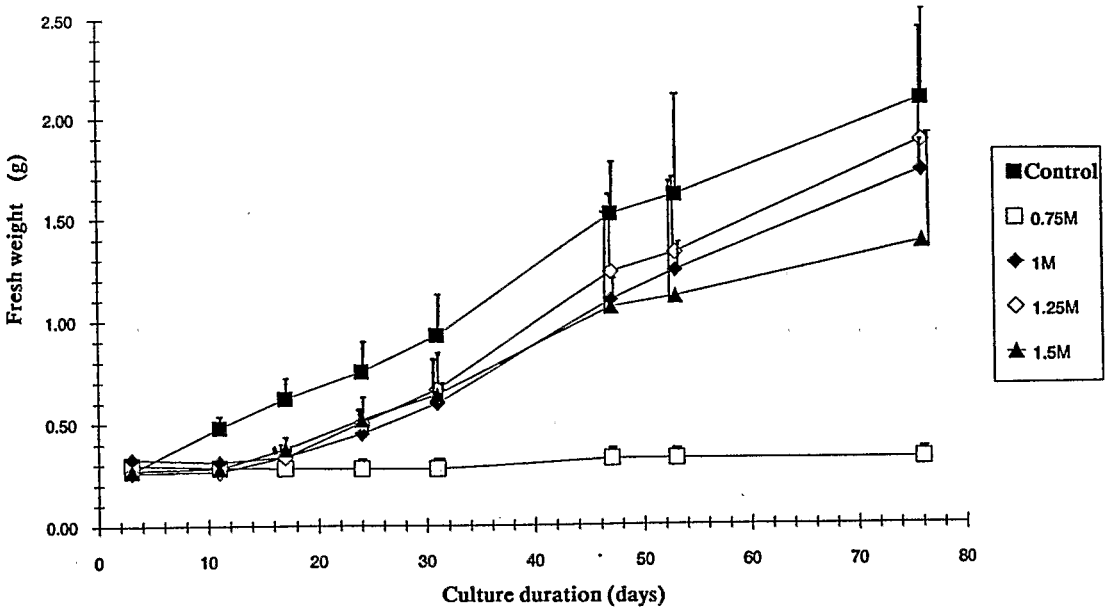

Fig.2: Fresh weight increase of untreated control and cryopreserved soybean calluses, after a 24-hour pregrowth duration with various sucrose concentrations. Vertical bars represent the standard deviation.

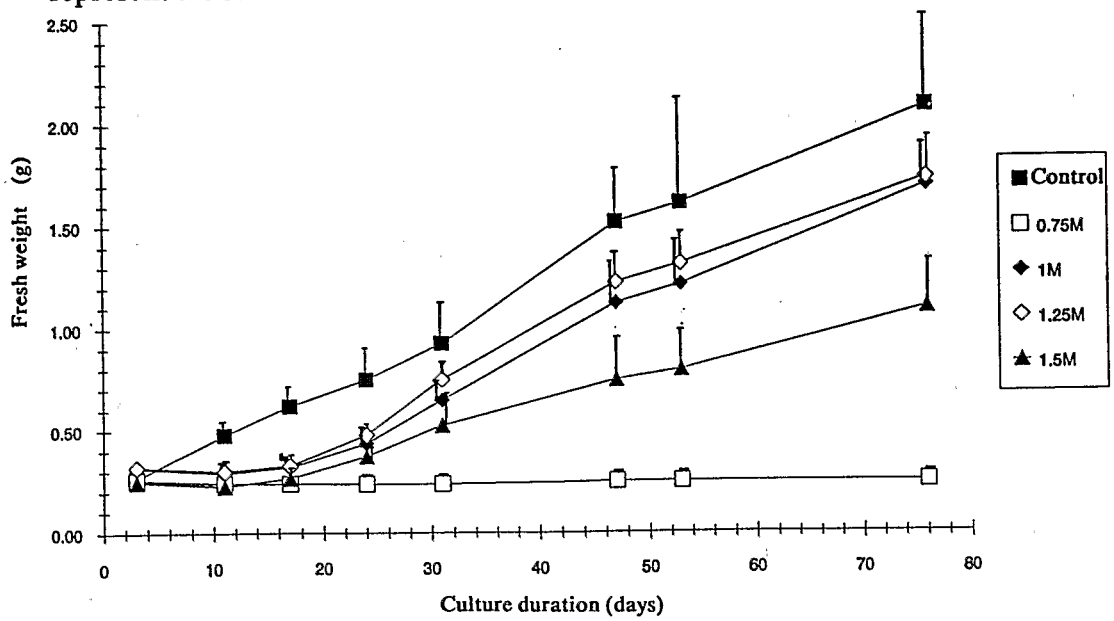

Fig.3: Fresh weight increase of untreated control and cryopreserved soybean calluses, after a 48-hour pregrowth duration with various sucrose concentrations. Vertical bars represent the standard deviation.