THE EFFECT OF MICROGRAVITY ON THE DEVELOPMENT OF PLANT PROTOPLASTS FLOWN ON BIOKOSMOS 9

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ABSTRACT

An experiment using plant protoplasts has been accepted for the IML-1 mission to be flown on the Space Shuttle in April 1991. Preparatory experiments have been performed using both fast and slow rotating clinostats and in orbit to study the effect of simulated and real weightlessness on protoplast regeneration. Late access before launch to the space vehicles has required special attention since it is important to delay cell wall regeneration until the samples are in orbit. On a flight on Biokosmos 9 ("Kosmos-2044") in September 1989 some preliminary results were obtained. The growth of both carrot and rapeseed protoplasts is decreased by 18% and 44% respectively, after 14 days in orbit compared to the ground control. The results also indicate that there is less cell wall regeneration under micro-g conditions. The production of cellulose in rapeseed and carrot flight samples was only 46% and 29% respectively of the ground controls. Also in both rapeseed and carrot, the production of hemicellulose in the flight samples was 63% and 67% respectively of that of the ground controls. At present both samples have reached the stage of callus development The peroxidase activity was also found to be lower in the flight samples than in the ground controls. Also the number of different isoenzymes was decreased in the flight samples. In general the regeneration processes are retarded in the flight samples with respect to the ground controls. From a simulation test experiment for IML-1 performed in January 1990 at ESTEC, Holland, regenerated plants have been obtained. These results are discussed and compared to the results obtained on Biokosmos 9.

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Biokosmos 9. Protoplast regeneration did not develop beyond the callus stage in either the flight or the ground control samples from the Biokosmos 9 experiment.

INTRODUCTION

Within the field of space cell biology the following groups of plants have been tested under microgravity conditions:

- unicellular plants
- callus and cells cultivated on solidified nutrient media
- protoplasts.

In space studies the unicellular algae Chlamydomonas reinhardii was studied on the Spacelab Dl- mission. The aim was to study the biological clock and other cellular functions - swimming velocity, proliferation, viability and ultrastructural alterations /1/. Callus and cell cultures can grow for an almost unlimited period at the cellular stage on solidified media as long as the cells have a satisfactory nutrient supply. The use of such cell types in space biology dates back to the joint US-USSR studies on earlier Soviet Biokosmos missions. On the unmanned satellite missions Kosmos 782 and 1129, experiments with free carrot cells demonstrated that the totipotent cells could give rise to carrot embryos which later on Earth developed into normal plants /2/.

The use of protoplasts in space biology studies is still in its infancy. Protoplasts are plant cells isolated from intact plant organs (stems, leaves, roots) by enzymatical treatment which removes the cell wall. Immediately after the removal of the walls the protoplasts start regeneration of new cell wall material. This is followed by cell division and formation of small cell aggregates within the first 5-8 days after the isolation of the protoplasts. The time period is dependent on the species. The aggregates develop further into callus tissue, embryos and eventually into mature plants.

The present paper describes the results from a preliminary experiment with rape and carrot protoplasts, placed on board Biokosmos 9 for a 14-day stay in orbit. This study was a link in the preparation for the IML-1 mission when a similar type of experiment will be performed. The results from a simulated space experiment performed at ESTEC in January 1990 will also be presented and discussed in correlation with the Biokosmos 9 results.

METHODOLOGY

Protoplast isolation

The protoplasts were isolated from 5 day old sterile cultivated, dark-grown hypocotyls of rape {Brasssica napus) and from carrot cell suspensions.

After preplasmolysis for 30 min, the rape hypocotyls were incubated for 20 hours with Cellulysin (1%) and Macerozyme (0.5%) at 25° C in the dark. The resulting protoplasts were washed and suspended in A-medium with a final concentration of 5.0×10^4 protoplasts per ml medium. For more details about the isolation procedure see Rasmussen et al. /3/. Carrot protoplasts were isolated after digestion in 5 ml 1% Cellulase Y and 0.1% Pectinase under the same conditions as described for rapeseed. After isolation, C^{14} - labelled glucose was added to some of the samples.

Hardware and cultivation on Biokosmos 9

A plant chamber (Figure 1) has been developed for the cultivation of the protoplasts onboard the Biokosmos 9 and the Space Shuttle. Each chamber contains 8 small polyethylene plastic bags. The opening is sealed with a silicon rubber membrane in a stainless steel fitting. Protoplasts and medium are loaded and withdrawn with the aid of a sterile syringe. The bags are completely filled with liquid medium (0.6 ml) and consequently there is no free liquid surface. Oxygen diffusion has to take place through the polyethylene membrane. The chamber fits into an ESA Type I container (Figure 1).

In addition the protoplasts were also cultivated in the Dynamic Cell Culture System (DCCS)

developed at ETH, Zurich, originally for the study of lymphocytes. The DCCS chamber was filled with 200 µl protoplast solution.

After isolation at IBMP in Moscow, the protoplast samples were placed in cultivation medium in the plastic bags and the DCCS-chamber and kept at 4° C in a specially constructed trolley (Figure 3) for transport from Moscow to Plesetsk, where the satellite (Figure 2) was launched. The container was provided with a temperature recorder.

During the orbital period the temperature in the biosattelite was kept at 23° C, but a temporary increase from 23° C to 27/28° C on ground was not compensated for in the biosattelite. However, tests performed have shown that an increase in that temperature range does not effect the developmental processes occurring during the first two weeks after protoplast isolation.

Ground controls identical to the flight samples were kept in an incubator under conditions similar to the flight conditions which were recorded continuously by telemetry channels. The main difference between the flight and the "synchronous" control samples was that the latter were delayed by 24 hrs.

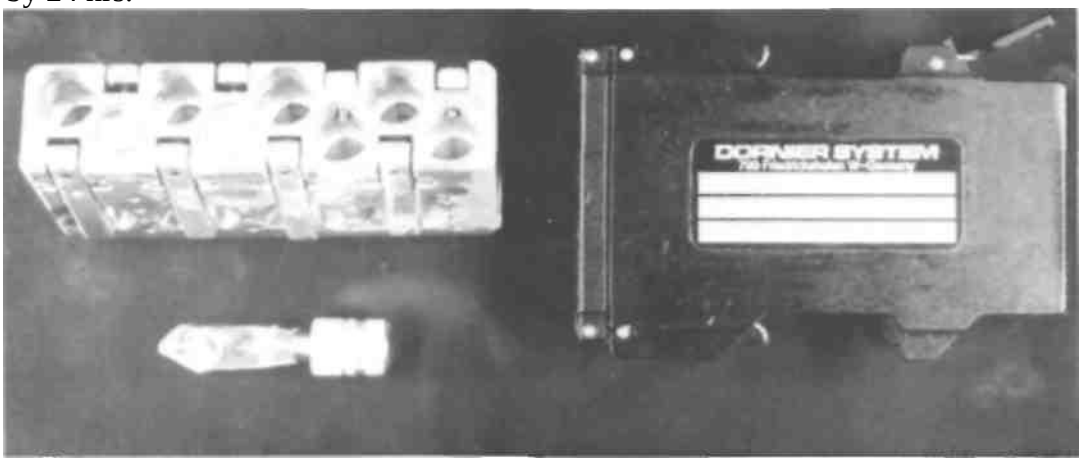

Fig. 1 Growth chamber for cultivation of protoplasts. The chamber fits into an ESA Type I container. Insert: Polyethylene bag with injection fitting.

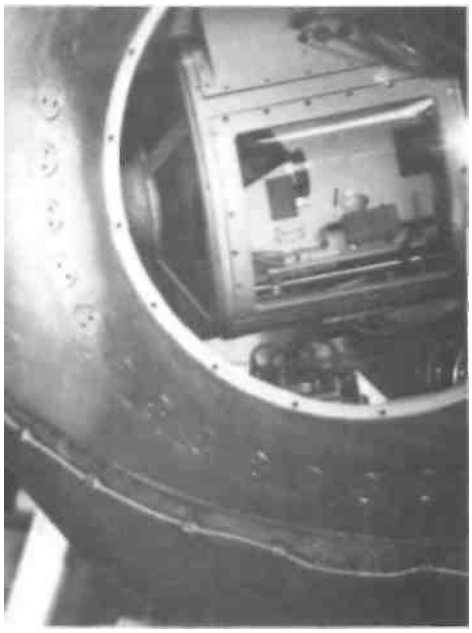

Fig. 2 The Biokosmos-satellite.

Fig. 3 The loading, in Moscow, of the samples into the ESA -trolley.

Cell culture regeneration and sample preparation

After retrieval, the regenerated protoplast cultures were immediately transported to Moscow at 4° C and initial tests on the material were started 12 h after landing. Some of the protoplasts were transferred to Petri-dishes and further cultivated in liquid A medium containing plant growth regulators (2, 4-dichlorophenoxyacetic acid, 6-benzylaminopurine and 1-naphthaleneacetic acid) in Trondheim. The growth of the rest of the samples was determined by measurements of the packed cell volume (PCV), in a graduated capillary tube, centrifuged for 5 min at 200g, followed by the determination of the total mass volume. Subsequently the samples were either fixed for microscopy or frozen for the analysis of isoenzymes and cell wall composition.

Light and electron microscopy

The samples of the tissues were fixed immediately after retrieval and later at different developmental stages for analysis by light (LM) and transmission (TEM) electron microscopy. The samples were fixed in 4% buffered glutaraldehyde, followed by a postfixation in 2% OSO_4 following standard methods.

Composition of the cell wall

The following major components were determined: pectin, cellulose, hemicellulose and intracellular polysaccharides. After separation and purification procedures the amounts of the different components were determined in relation to the glucose content in the aliqout.

SDS PAGE and peroxidase isoenzyme analysis

The retrieved samples were analysed using SDS polyacrylamide gel electrophoresis and silver staining for proteins. The peroxidase activity and isoenzyme pattern analysis were performed using IEF- isoelectrofocussing.

RESULTS AND DISCUSSION

Viability of plant cells is not influenced by microgravity conditions

The analysis of the samples retrieved from the satellite started 12 hours after landing. The initial analysis of viability of the cell aggregates using fluorescein diacetate (FDA) showed that more

than 50% of the total number of cells were still alive in the flight samples; the equivalent number in the ground controls was approximately 65% for both species (Table 1).

TABLE 1 Viability test using FDA.
3 lots of 100 protoplasts each were counted from each sample.

FLIGHT	Rapeseed	52%
	Carrot	53%
GROUND CONTROL	Rapeseed	65%
	Carrot	65%

The viabilities were similar to those of protoplasts obtained under ordinary laboratory conditions. The initial viability rates for rapeseed and carrot, were in the range of 90-95% immediately after the isolation of the protoplasts. However, the viability decreased during and after the initial cell division processes.

Cell proliferation decreases in orbit

The main parameter determined after retrieval of the protoplasts was the growth of the cell aggregates. The method of the packed cell volume (PCV) determination revealed a dramatic decrease in the cell proliferation under microgravity conditions. As shown in Table 2 a highly significant decrease in the growth of rapeseed cells (44%) and a similar but not significant decrease in carrot cells (18%) was observed after the 14 day orbital cultivation period.

TABLE 2 Cell growth during cultivation in microgravity for 14 days - packed cell volume (PCV) in µl, ± SD and Student-t analysis.*; significant difference at the 0.5% level. Number of samples = 6.

FLIGHT		PCV (µl)	% decrease
	Rapeseed	9.2 ±1.07*	44%
	Carrot	9.2 ±2.32	18%
GROUND CONTROL	Rapeseed	16.4 ±2.19*	
	Carrot	11.2 ±1.86	

At present such a decrease of cell growth under microgravity conditions is difficult to explain. However, during the IML-1 flight, a l-g control centrifuge will be part of the equipment on board. This will make it possible to distinguish the real microgravity effects from other pertubations, and show whether, indeed, microgravity causes decreased cell growth and/or proliferation. The results with these eukaryotic cells differ from observations made by Mergenhagen and Mergenhagen /1/ who found an increase in biomass yield of Chlamydomonas by exposure to micro-g during the D-l mission. Similar observations were made in Paramecium aurelia /5/ and Bacillus subtilis 161 - both species showed an increased cell proliferation in space.

Protein production and peroxidase activity are influenced by microgravity

Protein determination in the plant tissue immediately after retrieval revealed that the total protein production was decreased after the orbital period (Table 3A). A 71% decrease in protein was found in the rapeseed flight sample; the small total cell volume of the carrot tissue formed in

flight made it impossible to determine the amount of proteins by the methods used.

Also a decrease in the peroxidase activity was observed in the flight samples compared to the ground controls (Table 3B). The decrease was slightly different in the rapeseed and carrot samples - 56% and 47%, respectively, when measured as enzymatic activity per cell volume unit. Preliminary results from a protein analysis using SDS PAGE showed that distinct bands were missing in the flight samples compared to the ground controls. IEF-analysis also indicated differences between the isoenzyme pattern of peroxidases in the flight and ground samples. The latter observation is important since peroxidases are involved in the metabolism of plant cell wall material. It should be mentioned in this context that also Crowles et al. /7/ observed a reduced peroxidase activity in pine seedlings under microgravity conditions.

The biosynthesis of cell wall material is affected under microgravity

Before launch C^{14}-glucose was added to each of 3 samples of the flight and ground controls. After retrieval using conventional isolation and purification procedures, the content of pectin, cellulose and hemi-cellulose was determined. Calculations of all components were made relative to the content of glucose in the aliquot. The results obtained are presented in Table 4. Significant decreases were observed both in rapeseed and carrot - the content of hemicellulose decreased in both species with approximately 35%. The cellulose content was decreased by 71% in carrot and 54 % in rapeseed while only minor changes were detected in the pectin content.

TABLE 3 Protein (A) and peroxidase measurements (B) from representative samples. Proteins were determined by standard Lowry's method. Number of samples = 3.

A.

FLIGHT		Protein (µg) /PCV(µl)	Decrease
	Rapeseed	0. 27	71 %
	Carrot	< 0. 01	100%
GROUND CONTROL	Rapeseed	0.93	
	Carrot	0.64	

B.

FLIGHT		Peroxidase/PCV(µl)	Decrease
	Rapeseed	15	56%
	Carrot	8	47%
GROUND CONTROL	Rapeseed	34	
	Carrot	15	

TABLE 4 Cell wall fractions - C^{14} detection (mM glucose), SD = standard deviation of the mean.

RAPESEED	PECTIN	HEMI-CELLULOSE	CELLULOSE

FLIGHT	2.25 ± 0.32	0.26 ± 0.02	0.54± 0.08
CONTROL	2.20 ± 0.35	0.41 ± 0.03	1.17 ±0.15
% DIFFERENCE	+ 2.3%	-37%	-54%
CARROT			
FLIGHT	1.21 ±0.11	0. 08± 0.01	0.15± 0.02
CONTROL	1.25± 0.12	0. 12± 0.02	0.52± 0.06
% DIFFERENCE	- 3.2%	-33%	-71%

The lignin content has previously been found to decrease under microgravity conditions /7/. In the present study the young cell aggregates do not produce lignin and analysis of the lignin biosynthesis was therefore not performed.

Growth and differentiation of the cell cultures

After retrieval, rapeseed samples of flight and ground material not used for the immediate analysis described above, were transported from Moscow to Trondheim for further cultivation and regeneration. During transportation the environmental conditions were not optimal and microbial infection occurred in the samples. However, shortly after arrival in Trondheim, this was eliminated by using an antibiotic which had previously been shown to have no effect on the growth of the plant cells as such.

Since 29 September 1989 the cells have been growing in Petri dishes initially in liquid medium, at a later stage in solidified nutrient medium under standard laboratory conditions for cultivation of rapeseed tissue cultures. Figure 4 shows the developmental stage of the culture photographed in April 1990. As can be seen the flight sample is generally smaller than the ground control. Differentiation in plants has not yet taken place. In July 1990 the ground control was growing vigourously, but the flight sample had entered a dormant stage.

For comparison Figure 5 shows the developmental stage of rapeseed protoplasts originally isolated in January 1990 as part of a 12-day simulation experiment for IML-1 at ESTEC. The original protoplasts have developed into callus cultures and further into seedlings. It is difficult to explain why the Biokosmos 9 protoplasts have not yet differentiated into mature plants. This is not an effect of the microgravity conditions since the ground controls also behave in the same manner.

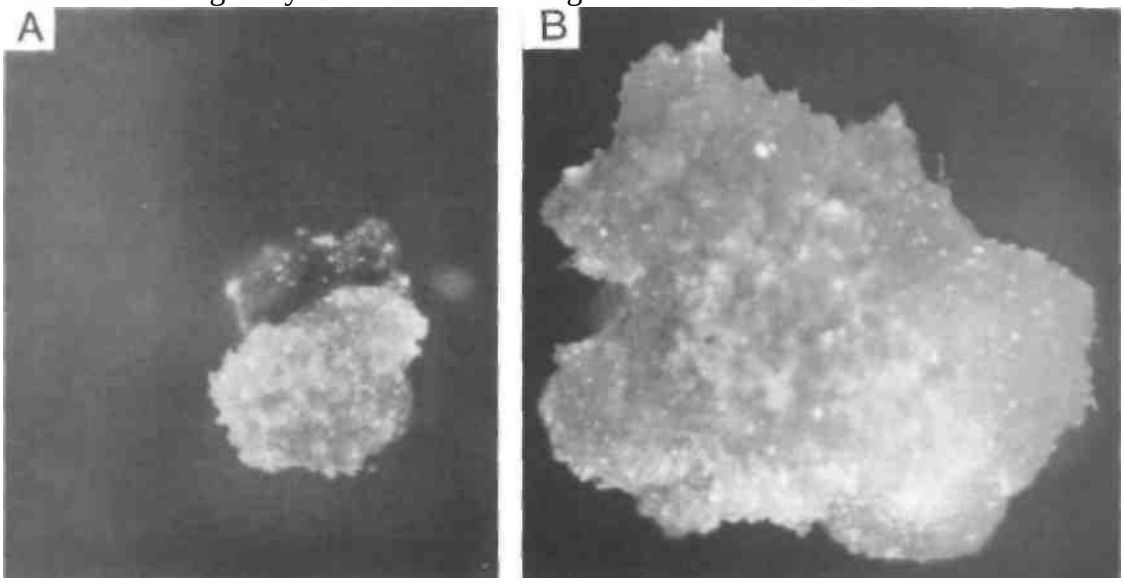

Fig. 4 Rapeseed callus tissue (age: 12 weeks) regenerated from protoplasts. Note the difference in total mass production between the flight sample (A) and the ground control (B).

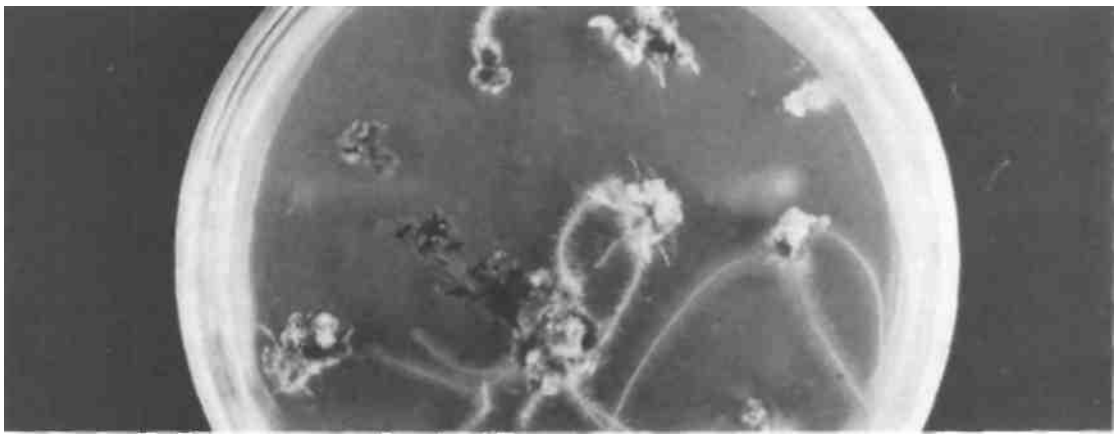

Fig. 5 Plant regenerated from rapeseed protoplasts via callus cultures (age: 4 months). The protoplasts were originally isloated for a simulated IML-1 test under laboratory conditions.

CONCLUSION

This preliminary experiment on Biokosmos 9 indicates a decrease in plant cell growth under microgravity conditions. All the results obtained so far indicate that in general the developmental processes are retarded under micro-g conditions. This is reflected in decreased and delayed production of cell wall components, changes in isoenzyme patterns and submorphological changes. The use of a l-g centrifuge on the IML-1 mission on the Space Shuttle together with the opportunity to take frequent samples during the flight period, will make it easier to draw a final conclusion about the influence of gravity on developmental processes during the regeneration of plant protoplasts.

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