STRUCTURAL AND FUNCTIONAL ORGANISATION OF REGENERATED PLANT PROTOPLASTS EXPOSED TO MICROGRAVITY ON BIOKOSMOS 9

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ABSTRACT

Preparatory experiments for the IML-1 mission, using plant protoplasts, were flown on a 14-day flight on Biokosmos 9 ("Kosmos-2044") in September 1989. Thirty-six hours before launch of the biosatellite, protoplasts were isolated from hypocotyl cells of rapeseed *(Brassica napus)* and suspension cultures of carrot *(Daucus carota).* Ultrastructural and fluorescence analysis of the cell aggregates from these protoplasts, cultured under microgravity conditions, have been performed. In the flight samples as well as in the ground controls, a portion of the total number of protoplasts regenerated cell walls. The process of cell differentiation and proliferation occuring in micro-g did not differ significantly from that observed under stationary conditions. However, differences were observed in the ultrastructure of the energetic organelles (plastids and mitochondria). There was also an increase in the frequency of the occurence of folds formed by the plasmalemma together with an increase in the degree of complexity of these folds. In cell cultures developed under micro-g conditions, the calcium content tends to decrease, compared to the ground control. Different aspects of the use of isolated protoplasts for clarifying mechanisms of the biological effect of microgravity are discussed.

INTRODUCTION

A number of space in vitro experiments with cultures of animal and plant cells, carried out during the recent decade, have demonstrated that cell metabolism is sensitive to microgravity e. g. an increased biomass production has been found in anise supsension cultures /1/ and a generally intensified secretion of physiologically active substances /2, 3/. Therefore it is a prerequisite for the development of future space biotechnology to increase our understanding of the basic gravity depending processes which occur in in vitro plant cultures. It is already established that changes occur in the thickening processes and the compositon of cell wall material in higher plants growing and developing under micro-g /4/. The rigid cell wall gives the plant cell a definite shape and strength and protects the cell protoplasm from environmental effects to a certain extent. The cell wall is also a prerequisite for cytokinesis.

Protoplasts are plant cells isolated from the plant tissue by an enzymatical treatment which removes the cell wall. After the artificial cell wall degradation a regeneration of new wall material follows immediately. Thereafter cell division starts and within the first 5 - 8 days, dependent on the species, after the isolation of the protoplasts the formation of small cell aggregates has ocurred. The aggregates develop further into callus tissue, embryos and eventually into mature plants.

Protoplasts represent a convenient model system for a comparative study of the structural and functional aspects of cell wall formation under stationary growth conditions and under the effect of extreme environmental factors. This has been the basis for the "Protodyn"-experiment where protoplasts from two angiosperm species have been cultured under micro-g conditions for 14 days onboard the "Kosmos-2044" biosatellite. After retrieval, biochemical /5/ and cytological

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analysis have been performed using the cell cultures developed. In the present studies results from the ultrastructural analysis are presented.

METHODOLOGY

The protoplasts were isolated from 5 day old sterile cultivated, dark-grown hypocotyls of rape (Brasssica napus) and from carrot (Daucus carota) cell suspensions - for details on the procedure see /5/.

The plant chamber used for cultivation on Biokosmos 9 has been described elsewhere /5, 6/. In short it consists of a chamber which contains 8 small polyethylene plastic bags which fit into a ESA Type I container. Protoplasts and medium - totally 0.6 ml - are loaded and withdrawn into the bags using a sterile syringe Oxygen diffusion takes place through the polyethylene membrane. After isolation of the protoplasts at IBMP in Moscow, the protoplast samples were kept at 4° C in a specially constructed trolley for transport from Moscow to Plesetsk where the Kosmos - 2044 satellite was launched. 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.

After retrieval, the regenerated protoplast cultures were immediately transported to Moscow at 4° C and samples were taken and fixed for electron microscopy 12 h after landing. The tissue was fixed in 2.5% glutaraldehyde in a 0.1M cacodylate buffer (pH 7.2) for 24 hours. Thereafter the cell suspension was washed thoroughly and embedded in 1.2% melted agarose at 38-40°C. The embedded samples were postfixed in 1% OsO_4 for 2 hours. Standard dehydration was performed in ethanol of ascending concentrations and acetone followed by embedding in a mixture of Epon and Araldite. Sections were cut on a LKB ultramicrotome and stained with lead-citrate and uranyl-acetate before examination in JEOL-100B and JEOL-1200 EX transmission electron microscopes. The Ca2+ content in the cells was determined by means of a Luman U3 microscope within a 530nm spectral range. The diameter of probes for analysis of the cytoplasm and the cell walls was 0.2 and 0.07mm, respectively. After fixation in 2% paraformaldehyde the material was washed for 1 hour and stained with 10-4M chlorotetracycline.

RESULTS

Freshly isolated rapeseed protoplasts are shown in Figure 1A. The cell wall is completely removed and cytoplasmic trabeculae can be observed on the inside of the plasmalemma. After retrieval the 14-day old rapeseed cell aggregates were photographed (Figure 1B, C). The flight sample is generally smaller than the ground control due to a delay in the growth of the flight sample compared to the ground control, but both cultures appear to grow well /5/. Only minor parts of the cultures show dark-coloured and degrading cells. Clear symptoms of ageing were also manifested by single cells surrounded by a wall but which had not started to divide. The presence of aggregates consisiting of 2-3 cells that had stopped their division and begun ageing was also observed. Actively dividing cells had formed colonies of 6 or more cells in the 14-day old cultures. In the flight variant at least a certain amount cells in colonies of 6 or more cells appeared to retain their ability for further proliferation. This was later demonstrated to be the case when the cultures were further cultivated in the laboratory /5/. In the control samples the ageing processes seemed to be more dominant and the number of cells capable of further proliferation was less. Main attention was therefore paid to a comparative study of the ultrastructure of the control and flight samples in colonies of 6 cells or more - both for rapeseed and carrot cells.

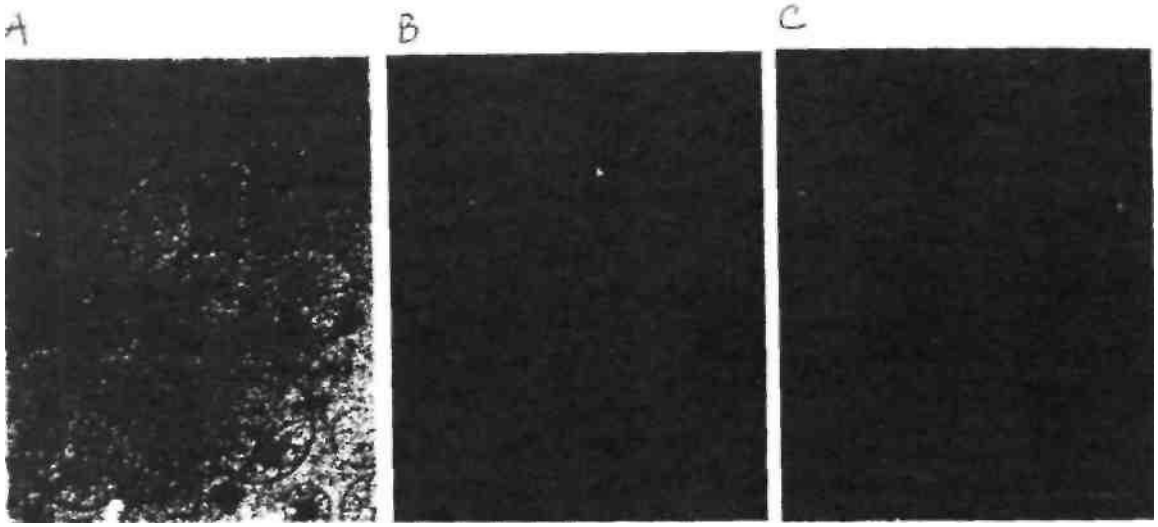

Fig. l. Freshly isolated rapeseed protoplasts (A) and the dividing cell aggregates obtained after 14-days in micro-g (B) and on the ground (C).

Ultrastructure of the 14-dav old rapeseed cell cultures

The newly isolated rapeseed hypocotyl protoplasts retain the features of the differentiated cells. During the experimental 14-day period the protoplasts as well as cells that regenerated walls but did not start division or cells that had stopped dividing, often contained large round plastids with starch grains. In the proliferating flight cells (Figure 2A) the number, size and structure of the plastids vary; hydrolysis of reserved starch occurs which results either in a size decrease or in disappearance of the starch grains. The number of smaller, round, elongated, pear-like and bent-shaped plastids rises (Figure 2A II, III) - dimensions 1-1.5 x 0.4-0.7μm. In the ground control cells the plastids averaging 2 x l.5 μm more often contained starch grains (Figure 2B I). Mitochondria in the control samples (Figure 2B I) were mainly round or oval, 1.2 x 1.0 μm on the average in dimension and with a more electron dense matrix than the flight sample. Mitochondria from the migro-g exposed cells (Figure 2A) were round (diam 0.3 μm), oval (O. 55 x 0.35 μm), elongated (up to 1.5 μm along the long axis) or of branched shape.

In bom the flight and the control sample the endoplasmic membrane system appears normal and well developed; the same applies to the microbodies. Lipid droplets are variable in size in the two samples - the average diameter in the flight sample is 0.65 μm; in the control cells the diameter is 1.2 μm. The plasmalemma is more or less smooth and tortuous, the periplasmatic space width varies in the ground control and the flight sample. Significant differences in cytokinesis between the control and flight samples were not observed.

The colony outer cell wall of me rapeseed control sample was on the average 0.67 μm thick; the wall between the neighbouring cells was 0.90 μm. The respective numbers for the flight sample were 0.27 and 0.30 μm. The intensity of the Ca^{2+}-luminescence in relative units are given in Table 1. As can be seen a significant decrease in the Ca-content occurs and can be detected both intracellularly and in the cell walls under micro-g conditions.

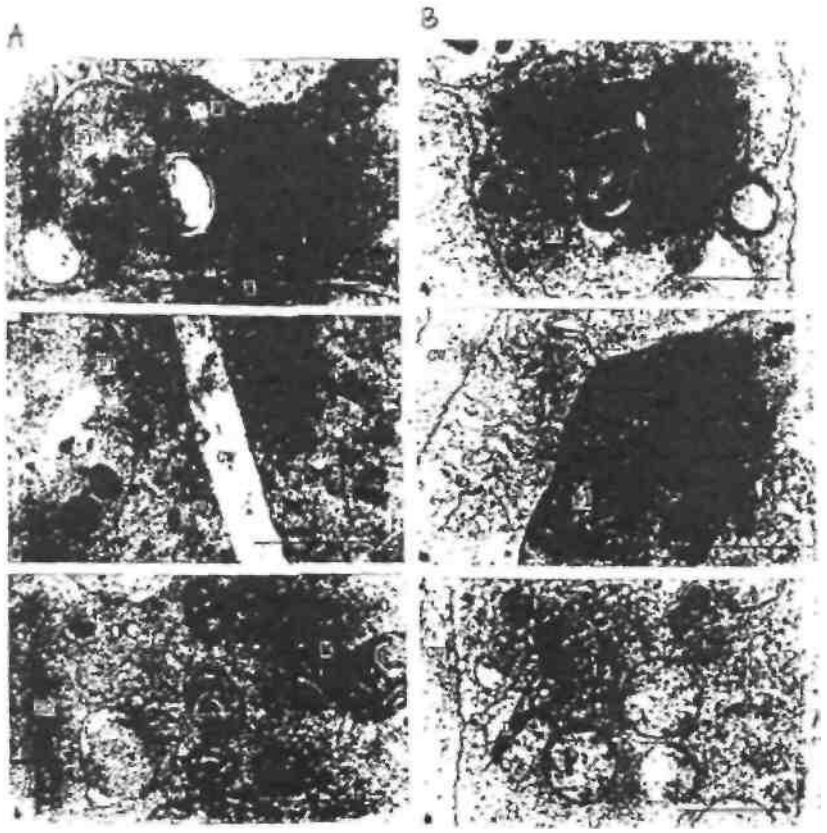

Fig. 2. The ultrastructure of 14-day old cell aggregates of the rapeseed flight sample (A) and ground control (B). The following organelles are indicated; N- nucleus, No - nucleolus, Pl - plastid, CW - cell wall, M- mitochondria, ER- endoplasmic reticulum, D- dictyosomes, LB - lipid body.

TABLE 1 The Ca^{2+} content given in relative units in the flight sample (F) and ground control (C) rapeseed and carrot cells.

	RAPESEED		CARROT	
	F	C	F	C
CELL CYTOPLASM	15.96 ± 1.3	29.56 ±2.1	16.80 ± 1.7	20.60 ± 1.8
CELL WALL	1.35 ±0.1	1.77 ±0.1	1.50 ±0.1	1.50 ±0.1

Ultrastructure of the 14-dav old carrot cell cultures

The same observation was made for 14-day old carrot flight and ground control samples as for rapeseed - the structural/functional organisation in colonies of 6 cells or more was generally similar. Numerous polysomes and ribosomes were observed in the cytoplasm and both r-ER and s-ER areobserved and appear well developed (Figure 3). Longer cisternae of r-ER with a more dense content compared to the control were more often observed in the flight cells. They were often arranged more or less in parallel to each other and formed a continuous network with shorter and wider, often branched cisternae of the s-ER type. The latter is represented in sections by numerous vesicles. Round, oval, pear-like microbodies with a fine granular matrix occur normally in angiosperm cells in suspension culture. In the carrot flight sample the number of microbodies increased considerably compared to the control - this is correlated to the increased volume of r-ER. Plastids containing a variable number and size of starch grains, osmophilic

globuli and non-identified membrane -bounded inclusions of variable form were detected both in control and flight samples. The total volume of the inclusions, however, in the cells grown under micro-g was exceeded that of the control group. Differences in the form and shape of mitochondria or their internal structure were not detected. Lipid droplets are observed as a rule in association with the ER, dictyosomes and mitochondria. The size of the droplet in flight sample cells exceeds on average that in the control cells. The dictyosomes in flight and control cells consist of 5-6 narrow cisternae and no broadening in these was observed (Figure 3). The plasmalemma has a relatively smooth contour in both types of samples and fits tightly to the cell wall. No cases of disturbances in cytokinesis in the examined samples were found. The outer cell wall of the carrot control sample was on the average 0.48 μm thick; the wall between the neighbouring cells was 0.50 μm. The respective numbers for the flight sample were 0.48 and 0.40 μm. The intensity of the Ca^{2+}-luminescence in relative units is given in Table 1. As can be seen, a decrease in the Ca-content occurs intracellulary but not in the carrot cell walls under micro-g conditions.

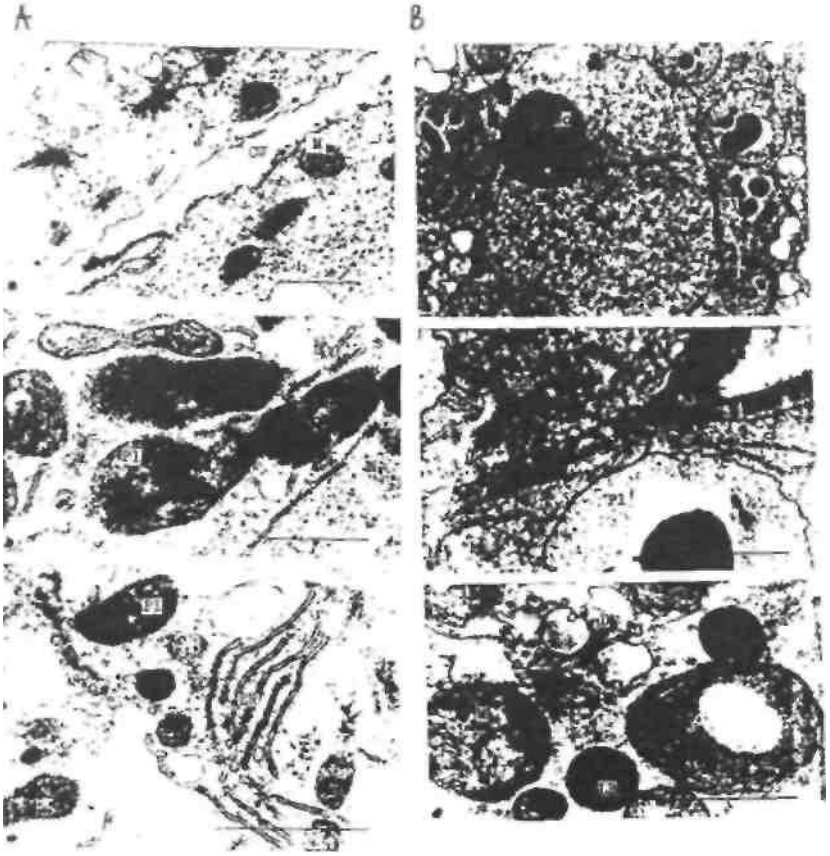

Fig. 3 The ultrastructure of 14-day old cell aggregates of the rapeseed *(Brassica napus)* flight sample. The following organelles are indicated; N- nucleus, No - nucleolus, Pl - plastid, CW - cell wall, M- mitochondria, ER- endoplasmic reticulum, D- dictyosomes, LB - lipid body.

DISCUSSION

The data presented in this study show that besides similarity in me ultrastructural organisation of meristem- and parenchyma-like cells of rapeseed and carrot grown under stationary conditions and in the orbital flight, certain differences from the ground control can be observed. The differences manifested as a result of the stay under microgravity conditions are an increased volume s-ER and r-ER, a higher electron density of the granular cisternal content, an increase in the lipid droplet volume and an increased frequency of membrane - bounded inclusions probably of protein nature. An increased production of microbodies was also observed in the flight sample of carrot.

In the flight samples of rapeseed the mitochondria shows a higher heterogenity than found in the control cells. In addition an increased occurrence of plasmalemma folds in the periplasmatic

space is also observed. The decrease in cell wall thickness, up to 2.5 - 3 times the control, can also be correlated to structural changes in the Golgi-apparatus under micro-g conditions.

The changes in wall thickness in rapeseed as a result of the stay in microgravity can also be correlated to the observed decrease in Ca-content of the cell wall. As shown in Table 1 a significant decrease is observed in the cell wall and a more dramatic fall is observed in the flight sample when compared with the Ca-content in the control cell cytoplasm. A similar decrease was also found in carrot cell cytoplasm but not in the cell walls (Table 2).

An increased volume of ER is observed during the process of cell wall regeneration in Skimmia /12/. It is predicted that the enzymes participating in the synthesis of cellulose precursors are formed in r-ER. The present data support this hypothesis. An increased ER volume under microgravity conditons, in particular in carrot, and a higher electron density of the r-ER cisternae, can be interpreted as an indication of adaptive stress in the micro-g environment. The predominance of aggregates of young cells under micro-g and those of ageing cells in the ground controls, find its explanation in a slowing down of the rate of cell wall regeneration under micro-g. This is further supported by the biochemical analysis of the same tissue - see Iversen et al. /11/. The explanation may partly be sought in the fact that fresh medium is not added during the 14-day cultivation period. In micro-g the delayed initiation of cell division processes results in aggregates of young and still dividing cells; the ground control cells have at a later stage of the cultivation period stopped cell division due to lack of nutrients and are undergoing ageing processes when the 14-day period is over (cf. Figures 2 and 3).

The special features in the ultrastructure of meristem-like carrot cells, in the first place observed as an increased population of microbodies probably with lysosomal activity and the increased thickness of the cell walls under micro-g indicate that carrot cells casilier adapt to these conditions than rapeseed cells. In this context it is important to stress that the carrot protoplasts originate from an undifferentiated cell suspension; the rapeseed from a highly organised and complex intact plant.

Similar observations were made using Haplopappus gracilis tissue cultures cultivated during a 8 -and 13 - day orbital flight (unpublished results).

The results of the ultrastructural analysis of the carrot cells in the present study are in agreement with the data obtained on somatic embryogenesis in carrot suspension culture growing for 20 days during an orbital flight /13, 14/. Embryoid formation under these conditions was similar to the control variant - in the after-flight period the embryoids developed into normal plants. The various stages of differentiation of the original plant material from which the protoplasts were isolated makes it difficult to make a direct comparison between rapeseed and carrot based on the results obtained in this study. The conclusion can, however, be drawn that microgravity modifies the ultrastructural development from protoplasts to cell aggregates in both species. A well documented effect is the 2.5 - 3 times decrease in wall thickness under micro-g in the 14-day old samples of rapeseed which can be correlated to the decreased content of cellulose and hemicellulose /5/. This can be interpreted as the result either of a decrease in synthesis or an intensification in hydrolysis of the cell wall components. At present, the mechanism of these processes and the triggers of their induction are still far from being elucidated. In order to solve the problems of gravisensitivity for basic cell processes in plants more effort has to be put into analysis of the type presented in this study. The use of protoplasts is an adequate model system for elucidating the problems of structural changes and cell wall differentiation under micro-g conditions. During the IML-1 flight in April 1991 on the Space Shuttle it will be possible to perform preservation of the experimental material under the orbital period. In addition the use of a l-g centrifuge makes the observations more reliable and their interpretation easier.

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