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Fusion and metabolism of plant cells as affected by microgravity

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Abstract. Plant cell protoplasts derived from leaf tissue of two different tobacco species (*Nicotiana tabacum* L., *N. rustica* L.) were exposed to short-term (sounding rocket experiments) and long-term (spacelab) microgravity environments in order to study both (electro) cell fusion and cell metabolism during early and later stages of tissue regeneration. The period of exposure to microgravity varied from 10 min (sounding rocket) to 10 d (space shuttle). The process of electro fusion of protoplasts was improved under conditions of microgravity: the time needed to establish close membrane contact between protoplasts (alignment time) was reduced (5 as compared to 15 s under 1 *g*) and numbers of fusion products between protoplasts of different specific density were increased by a factor of about 10. In addition, viability of fusion products, as shown by the ability to form callus, increased from about 60% to more than 90%. Regenerated fusion products obtained from both sounding-rocket and spacelab experiments showed a wide range of intermediate properties between the two parental plants. This was verified by isozyme analysis and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR). In order to address potential metabolic responses, more general markers such as the overall energy state (ATP/ADP ratio), the redox charge of the diphosphopyridine nucleotide system (NADH/NAD ratio), and the pool size of fructose-2,6-bisphosphate (Fru 2,6 bisp), a regulator of the balance between glycolysis and gluconeogenesis, were determined. Responses of these parameters were different with regard to short-term and long-term exposure. Shortly after transition to reduced gravitation (sounding rocket) ratios of ATP/ADP exhibited strong fluctuation while the pool size of NAD decreased (indicating an increased NADH/NAD ratio) and that of Fru 2,6 bisp increased. As similar changes can be observed under stress conditions, this response is probably indicative of a metabolic stress compensation. Samples taken for up to 7 d of exposure to microgravity showed the opposite effect. Here, the ratios of ATP/ADP and of NADH/NAD, and the pool size of Fru 2,6 bisp were decreased. We take this as an indication of metabolic relaxation, i.e. decreased metabolic turnover. As rates of protoplast regeneration and cell division were obviously similar to 1-*g* controls, we conclude that under conditions of microgravity regenerating tobacco mesophyll protoplasts need less metabolic energy for the same effort.

Key words: Adenylate – Electro fusion – Fructose-2,6-bisphosphate – Microgravity – *Nicotiana* (protoplasts) – Protoplast (regeneration) – RAPD-PCR – Redox charge

Introduction

Protoplasts can be isolated from various plant tissues by enzymatic digestion of the cell wall. They have become an important tool in many different research areas. Devoid of the rigid wall, protoplasts are easy to manipulate, and can be used for somatic cell fusion in order to recombine different genetic properties of sexually incompatible parental species. As rather homogeneous cell populations they are well-suited for physiological investigations, and due to their capability for regeneration, they are a convenient model system to study plant developmental processes.

With regards to electrical cell fusion (Zimmermann et al. 1984) there are some inherent physical and technical, as well as biochemical factors which can reduce the theoretically achievable yield of viable hybrids under terrestrial gravitation. These are movement of fusion partners due to convection, improper

Abbreviations: Fru 2,6 bisp = fructose-2,6-bis phosphate; P(+) = regular protoplasts (with vacuole); P(−) = protoplasts without vacuole (evacuolated); PAL = phenylalanine ammonia-lyase; RAPD-PCR = random amplified polymorphic DNA-polymerase chain reaction

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alignment due to sedimentation, or decreased viability of fusion products as a consequence of the need for higher than theoretically necessary membrane-breakdown voltages (Mehrle et al. 1989). These restrictions apply especially for the fusion of plant cell protoplasts which differ in diameter and specific density, and can be avoided when movement caused by convection or sedimentation does not take place. Such an environment is given under conditions of microgravity, and it has been shown that this can improve fusion rates of yeast (Zimmermann et al. 1985), animal (Zimmermann et al. 1988) and plant cells (Mehrle et al. 1989).

Gravity affects the orientation of plants in many ways (Volkmann and Sievers 1992a). It is, however, not clear whether each cell senses gravity in some way (for example via the suspension of organelles within the cytoskeleton) without showing a visible response, or if only specialized cells such as statocytes have the ability to sense gravity and participate in the conversion of changes in the gravity vector into a reorientiation of the respective organ. It is feasible to assume that within any cell sedimentation of organelles (nucleus, plastids, mitochondria) has to be conteracted by contractile elements of the cytoskeleton, a process which should consume energy in the form of ATP. Lack of sedimentation should thus cause less energy requirement to keep organelles positioned. This could affect cellular metabolism. Information on effects of reduced gravitation on cellular metabolism is very scarce. One consistent observation regarding metabolism of plants grown in microgravity or under horizontal clinorotation is reduced starch accumulation (compare Obenland and Brown 1994). Reduced starch levels could, however, not be correlated with altered extractable activities of key enzymes of starch and carbohydrate metabolism.

In order to address this topic, we decided to analyze a few parameters which give a more general characterization of the metabolic situation in a cell. From studies with regard to metabolic reponses to environmental stress we know that the cellular energy state offers a good marker. Cells showing stress metabolism have increased ratios of ATP/ADP (Weidmann et al. 1990) and of NADH/NAD (Einig et al. 1987), as well as an increased pool size of Fru 2,6 bisp (Einig and Hampp 1990), an important regulator of the balance between glycolysis and gluconeogenesis in plants (Stitt 1990). In this study we compare changes in these parameters in response to short-term (up to 10 min) and long-term exposure (up to 7 d) of protoplast suspensions to a microgravity environment, in addition to fusion parameters and yields of viable fusion products.

Materials and methods

Protoplast isolation. Mesophyll protoplasts were isolated from sterile shoot cultures of two tobacco species, *Nicotiana tabacum* L. cv. Xanthi and *N. rustica* L. by enzymatic digestion with 2% Cellulase Onozuka R 10 and 0.5% Macerozyme R 10 (both Serva, Heidelberg, Germany) in isolation buffer (0.6 M mannitol, 1 mM $CaCl_2$, 0.5% bovine serum albumin, 0.2% sodium ascorbate, 5 mM Mes, pH 5.6). After 2–3 h incubation at 30 °C, liberated protoplasts were collected by centrifugation (2 min, 200 *g*) and purified by flotation in a sucrose/mannitol step gradient (0.5 M sucrose or mannitol, 1 mM $CaCl_2$, 5 mM Mes, pH 6.0). Protoplasts of *N. rustica* were subsequently evacuolated by iso-osmotic density-gradient centrifugation (117 000 *g*, 30 min, 23 °C) in Percoll buffer [0.5 M mannitol, 100 mM $CaCl_2$, Mops at pH 7.0 in Percoll (Pharmacia, Freiburg, Germany)]. After two final washes in mannitol buffer, a 1 + 1 mixture of parental protoplasts (for fusion prior to culture) was adjusted to $2.5 \times 10^6 \cdot ml^{-1}$ in mannitol buffer. The suspension density for the individual protoplast types was $5 \times 10^6 \cdot ml^{-1}$ in culture medium (KM-medium; Kao and Michayluk 1975, modified according to Naton et al. 1992). The suspensions were stored at 4 °C until use.

Protoplast fusion and cultivation. Immediately prior to fusion, the protoplast suspension was diluted to $6 \times 10^5 \cdot ml^{-1}$ with fusion medium (0.5 M mannitol), and transferred into the fusion chamber which was inserted in a sample holder allowing microscopic observation (electrode distance 1.2 mm; Hoffmann et al. 1995b). Under optical control, fusions were performed after alignment at 20 V_{pp} (alternating current) and 2 MHz by a single direct-current fusion pulse of 100 V and 50 µs duration. The fusion mixture was withdrawn from the fusion chamber 10 min later and cultured at a final density of $1.2 \times 10^5 \cdot ml^{-1}$. The parental cell suspensions were diluted to $2.5 \times 10^5 \cdot ml^{-1}$ and incubated at 25 °C under microgravity or 1 *g* conditions.

Hardware and operational flow. Due to the limitations caused by telemetric experimentation during a sounding rocket flight and inside a spacelab, special sets of hardware were designed. These are detailed in Mehrle et al. (1989), Klintworth et al. (1994) and Hoffmann et al. (1995b).

Post-flight cultivation. The cell suspensions from the different cultivation chambers were transferred to petri dishes and cultivated further as described by Naton et al. (1992). Regenerated plants were maintained as sterile shoot cultures.

Isoenzyme analysis. The isoenzyme patterns of esterases and basic peroxidases were used to identify putative *N. tabacum* × *N. rustica* hybrids. For the esterase assay (Bates and Hasenkampf 1985, modified according to Naton et al. 1992), leaves of the regenerated plants were harvested and homogenized on ice in Tris-HCl buffer [0.5 M (pH 6.8) containing 2% (w/v) polyvinyl pyrrolidone (PVP), 80 mM dithiothreitol (DTT), and 0.2% Na_2SO_3, 0.15% $Na_2O_2S_5$, according to Stegemann and Sohnick 1982]. The extracts were centrifuged (10 000 *g*, 15 min, 4 °C), the supernatant supplemented with 7.5% (w/v) sucrose and frozen at –80 °C until use. To test for peroxidase activities, leaves were homogenized in Tris-phosphate buffer [0.26 M (pH 6.9) containing 10 mM DTT with 2% (w/v) PVP; Mäder et al. 1975), centrifuged, dialyzed against 1/10 diluted Tris-phosphate buffer overnight, and then immediately used for electrophoresis. For enzyme visualization, the procedures of Scandalios (1969) and Mäder et al. (1975) were modified according to Naton et al. (1992). Esterase activity was stained in 0.1 M phosphate buffer (pH 6.6) with 0.08% (w/v) Fast Blue RR and α-naphthylacetate [4% (v/v) of a 1% (w/v) stock solution in 70% ethanol]. After incubation for 10–30 min at room temperature (RT) in the dark, grey bands became visible on a clear background. Acid peroxidases were visualized by adding 1 vol. of substrate solution (0.25% (w/v) *o*-dianisidin and 0.15% (w/v) β-naphthol in acetone) to 4 vol. of 10 mM Tris-acetate buffer (pH 4.5) and starting the reaction with 30% H_2O_2 ($1\ \mu l \cdot ml^{-1}$). Incubation for 5–15 min at RT revealed greenish-brown bands on a light yellow background.

Fixation and light microscopy. Aliquots (0.5 ml) of the protoplast cultures were fixed in GA-buffer (3% glutaraldehyde in 0.2 M phosphate buffer, pH 5.7: Sörensen buffer). The cells were withdrawn from the fixation chambers with syringes, followed by two rinses with fresh GA-buffer. They were collected by centrifugation,

and counted under the light microscope in a Fuchs-Rosenthal haemocytometer.

Protein extraction and determination. The ethanol extracts (0.5 ml cell suspension mixed with 1.5 ml ethanol abs.) were removed from the fixation chamber. Precipitate and solvent were collected in an Eppendorf cup, and sonicated for 30 min on ice. The homogenized sample was centrifuged (15 min, 10000 *g*, 2 °C). The pellet was dried under vacuum and resolubilized in Laemmli buffer for SDS-PAGE (Laemmli 1970). The protein concentration was determined as described by Neuhoff et al. (1979) and Guttenberger (1991).

Genotypic identification of hybrids by the amplification of random genomic DNA by polymerase chain reaction (*RAPD-PCR*). Isolation of DNA from leaf slices or sliced callus as well as RAPD amplification are described in detail in De Filippis et al. (1996). Primers were from the UBC Set 50/4(1) (University of British Columbia, Vancouver, Canada). The largest number of bands were obtained with primer UBC-344 (5′TGTTAGGCAC 3′) in addition we employed primers UBC 335, UBC 337, and UBC 350 for statistical and dendrogram analysis. The resulting bands were scanned and evaluated by a computerized gel scanning system (Hirschmann, München, Germany; see below).

Polyacrylamide gelelectrophoresis (*PAGE*). The extracts were separated on a 10% acrylamide gel, run for 2 h at increasing voltage (10 min 60 V, 10 min 80 V, 80 min 100 V). The gels were 5 cm × 5 cm × 0.5 cm in size. The buffer system was according to Naton et al. (1992). From 2 to 3 µl of sample was loaded onto each lane, which corresponded to approximately 4 µg of protein.

For immunological studies electrophoreses were carried out in a microgel-system (Neuhoff 1982). The dimensions of the slab gels were 2.6 cm × 3.5 cm × 0.5 cm. The acrylamide concentration was 15% or 12%, with a 3% stacking gel. Electrophoreses were run for 30 min (10 min 60 V, 10 min 80 V, 10 min 100 V). The gels were fixed in 10% sulfosalicylic acid and stained with Coomassie Brillant Blue (Neuhoff et al. 1985), or were equilibrated in transfer buffer for Western blotting.

Immunoblotting. The protein was transferred from the gel to a nitrocellulose membrane by a semi-dry blot (Kyhse-Andersen 1984) with six layers of filters (No. 1; Whatman, Maidstone, UK) equilibrated in transfer buffer [25 mM Tris, 150 mM glycine, 10% (v/v) methanol, pH 8.3] on each electrode. Blotting was for 1 h at 120 mA. Immunostaining was done with the Bio-Rad (München, Germany) GAR-AP-Kit (secondary antibody: goat anti-rabbit, alkaline-phosphatase conjugate); instead of gelatine, defatted milk powder was used in the blocking solution (4% w/v) and the antibody buffers (1% w/v). The polyclonal primary anti-sera against tubulin and actin were purchased from Sigma (Deisenhofen, Germany). Densitometry was done with a Hirschmann Elscript 400 equipped with microscope optics.

Adenine nucleotides. Adenine nucleotides were determined in a luminometric assay according to Hampp (1985). It is based on the stoichiometric conversion of 1 ATP to 1 quantum of light by the luciferin-luciferase system (Lumit reagent from Perstorp, Rodgau, Germany). The ADP was measured in the same manner after enzymatic conversion into ATP by pyruvate kinase with Phosphoenolpyruvate as substrate. The assay was performed in microwell plates and had a sensitivity down to 0.2 pmol · $(10\ \mu l)^{-1}$ sample volume.

Pyridine nucleotides. Pyridine nucleotides were determined by enzymatic cycling (Lowry and Passonneau 1972). In the first reaction step (cycling reaction) the respective nucleotide serves as coenzyme in a coupled reaction system. Since it is continuously regenerated, it can engage in the same reaction several thousand times, accumulating an intermediate product. After terminating the reaction by heat treatment (15 min, 95 °C) this product is determined in a second step (indicator reaction), in which a stoichiometric amount of NAD(P)H is produced and quantified by photometry. As both oxidized and reduced forms are determined the same way, separation is via extraction; oxidized pyridine nucleotides are extracted in acid, reduced pyridine nucleotides in base. Heat treatment ensures the destruction of the respective counterpart.

Fructose-2,6-bisphosphate. The determination of Fru 2,6 bisp was based on the activation of the plant enzyme PPi-dependent fructose 6-phosphate phosphotransferase by very small amounts of this metabolite (Steingraber et al. 1988). In a reaction cascade this activity is coupled to the oxidation of NADH to NAD. Extracts in 0.1 M NaOH + 0.08% (v/v) Triton X-100 were heated to 80 °C for 5 min. After centrifugation (10 min, 10 000 *g*, 4 °C) the supernatant was taken directly for the determination of Fru 2,6-bisp. From 10 to 20 µl extract was added to 50 µl of the specific reagent mixture [200 mM Tris-acetate (pH 7.9), 0.04% (w/v) BSA, 2 mM fructose 6-phosphate, 0.5 mM NADH, 1 mM pyrophosphate, 30 µg · ml^{-1} aldolase, 0.4 µg · ml^{-1} triosephosphate isomerase, and 4 µg · ml^{-1} glycerol 3-phosphate dehydrogenase].The pH was kept slightly alkaline (7.5–8.2) and the assay performed in microplate wells. The decrease in NADH concentration was measured with a microplate reader system as above.

Results

Electrofusion. In order to faciliate the observation of the fusion process and the identification of fusion products immediately after electrofusion, the protoplasts from one parental species (*Nicotiana rustica*) were evacuolated [P(−)]. This results in smaller, dense, dark-green spheres compared to untreated mesophyll protoplasts (from *Nicotiana tabacum*), which still contained their vacuoles [P(+)]. Evacuolation also causes a considerable difference in specific density, which can be used to separate fusion products from unfused cells by density-gradient centrifugation. On the other hand, the rates of P(+)/P(−) heterofusions are low under 1 *g*, because one of the partners sediments faster than the other, resulting in particle separation. Under microgravity (sounding rocket experiments) a higher frequency of heterofusions was observed for tobacco (Mehrle et al. 1989; Hampp et al. 1992) and *Digitalis* protoplasts (Baumann et al. 1990; Hampp et al. 1990), combined with an increased viability of the fusion products, due to a reduced alignment-field strength and duration. Under extended duration of microgravity, as offered by spacelab, these observations could be repeated and extended to sunflower protoplasts (Table 1; see also Hoffmann et al. 1995b).

Protoplast development under microgravity. The development of the parental protoplast cultures during the 10-d flight was monitored by microscopy of the glutaraldehyde-fixed samples. As in normal laboratory cultures, the evacuolated protoplasts regenerated vacuoles, and, similar to regular mesophyll protoplasts, started to regenerate cell walls. The first divisions were observed on day 7. Figure 1 gives a survey of the data obtained from cell counting. It is quite evident that the overall development was not influenced by gravity conditions. Differences were within the range of variation between the individual cell chambers. Representative stages of development are shown in Fig. 2.

Table 1. Summary of electrofusion data from different flight opportunities and 1-*g* reference experiments. Data for sunflower and foxglove fusions are compiled from von Keller et al. (1994) and from Eisenbeiss et al. (1994), respectively

Parameter	1 *g*	microgravity	
Duration of	15	5	(tobacco)
dielectrophoresis (s)	15	7	(sunflower)
(= protoplast alignment)	7	4	(foxglove)
Number of pulses	1	1	(tobacco)
(50 μs; ≈ 1 kV/cm)	3	3	(sunflower)
	1	1	(foxglove)
Yield of fusion	1	13	(tobacco)
products (%)	15	27	(sunflower)
	0.7	7	(foxglove)
Viability of hybrids (%)	50–60	>90	(tobacco)

The microcalli which had developed under microgravity were cultivated further on ground. They were plated on agar medium and were transferred several times until the calli reached a size suitable for shoot induction (5–7 months). After successful organogenesis the shoots were cut and transferred to fresh medium for root formation. By this procedure, a total of 559 plants was regenerated from the flown samples. Table 2 gives a summary of the regeneration success for the different chambers. Values between 70 and 90% morphogenesis are the usual range for our tobacco protoplast cultures. Note that the example of a 100% morphogenetic capacity refers to a P(−) culture grown under microgravity. It can thus be concluded that in this experiment the tobacco protoplast cultures exhibited equal development under microgravity and 1 *g*.

Hybrid analysis. Products of cell fusion were either recovered immediately after fusion (sounding-rocket experiments) or cultivated under microgravity for about 10 d (spacelab) before plant regeneration under 1 *g* (ground laboratory) was initiated. Out of 55 plants tested (spacelab experiment), 11 showed an intermediate isoenzyme pattern when stained for esterase and peroxidase activity (Fig. 3). This would correspond to a frequency of 20% in the original suspension. The same phenomenon was found in laboratory reference experiments, with, for example, 13% of hybrids recovered after an initial heterofusion yield of only 2%. This may indicate a selective advantage of the hybrids generated under microgravity during further regeneration, like faster callus growth or earlier morphogenesis.

Some of the somatic hybrids were analyzed by RAPD-PCR. Separation of the products of random amplification on polyacrylamide gels delivered a clear distinction of parental plants from the six hybrids tested (Fig. 4). A detailed analysis of pherograms derived from silver-stained gels shows that the somatic hybrids inherited DNA bands from both parents, although to a variable degree. There was also a low percentage of bands which could not be assigned to a parent. A summary of leaf morphological features of the six hybrids, together with the respective RAPD band pattern inheritance is presented in Table 3 (see also De Filippis et al. 1996).

Protein analysis. The physiological condition of a cell is also reflected by its protein content, as shown by many

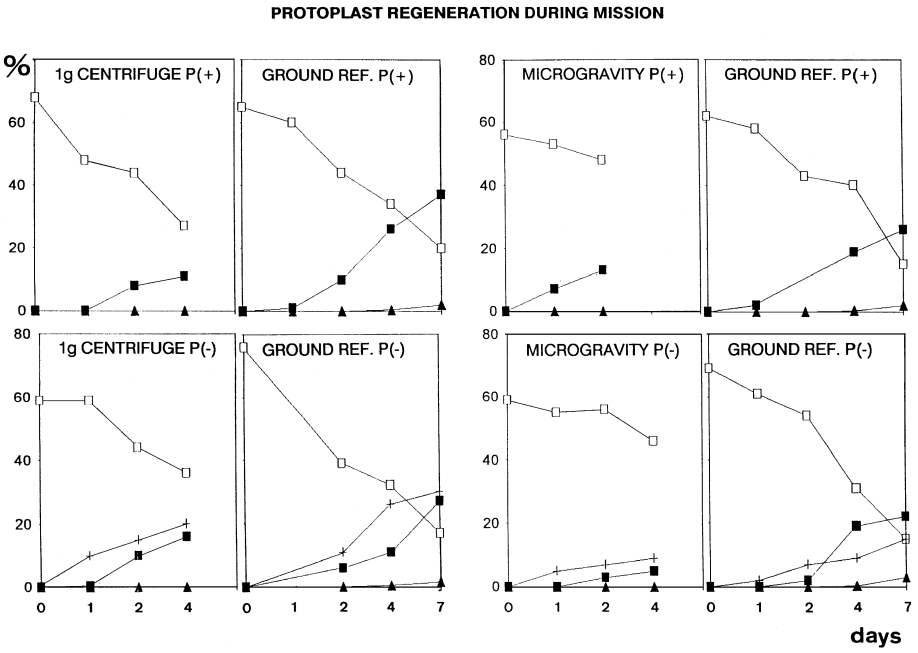


Fig. 1. Initial development of parental protoplasts, *Nicotiana tabacum* [P(+)] and *N. tabacum* [P(−)] in flight and ground cultures. Glutaraldehyde-fixed samples were used to count the different developmental stages: percentage of vital protoplasts (□), vacuolation (+), cell wall formation (■), and cell divisions (▲). The cultures contained 30–40% degenerating protoplasts

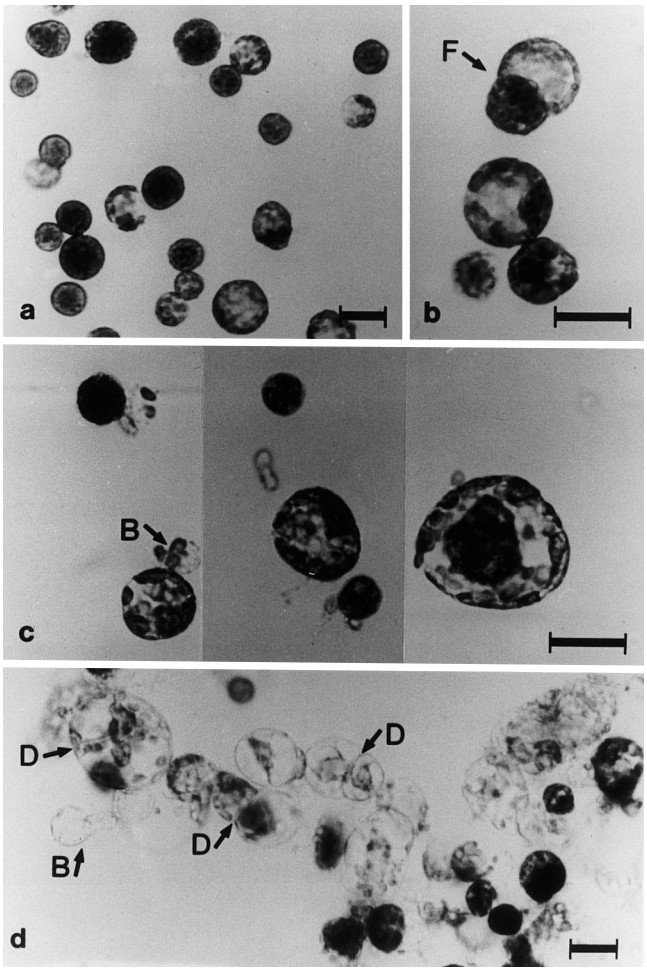


Fig. 2a–d. Glutaraldehyde-fixed samples of *Nicotiana* protoplasts showing representative developmental stages from flight cultures. **a** Overview of a mixed suspension with bright, vacuolated *N. tabacum* and dark, evacuolated *N. rustica* protoplasts. **b** Heterofusion product (*F*) immediately after fusion. **c** Cell wall formation as indicated by a change in cell shape and by budding (*B*). **d** The first division stages (*D*) occurred on day 7. Bars = 25 μm

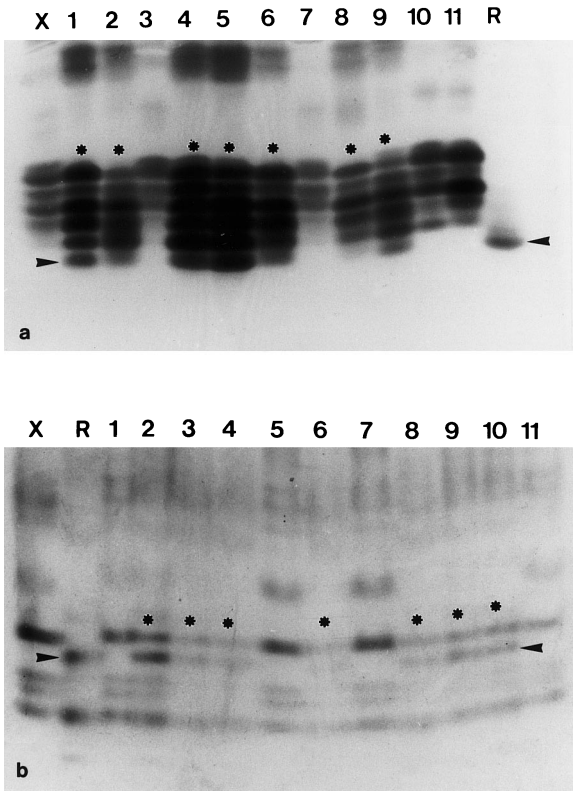


Fig. 3a,b. Isoenzyme analysis of acid peroxidases (**a**) and esterases (**b**). *Lanes X* and *R* show the parental patterns of *N. tabacum* Xanthi and *N. rustica*, respectively; *lanes 1–11* show extracts from regenerated plants. Hybrids were identified by the additional *N. rustica* bands (*arrows*) and are marked by *asteriks*

developmentally regulated polypeptides (e.g. Bernier 1988; Novak and Kohn 1991) or proteins which are synthesized in response to stress (Bowels 1990). Rasmussen et al. (1992) found a major decrease of total protein content and the disappearence of distinct bands from the electrophoresis pattern in cells grown under microgravity. Therefore, protein analysis was also performed with spacelab samples.

Due to rather heterogeneous preparation of aliquots under spacelab conditions (or problems in recovering the sample quantitatively from the fixation chambers), there was a high standard deviation for the protein values obtained from a single cell chamber. The average protein content, too, varied between the different cultures, but these differences were not significiant when tested by variance analysis (level of significance, 5%). The total cell protein pattern after SDS-PAGE, visualized by Coomassie Briliant Blue staining, is shown in Fig. 5. In

Table 2. Regeneration efficiency of plantlets from spacelab D-2 cultures. T = total number of propagated calli, G = percentage of green calli, D = percentage of dead calli, M = percentage of morphogenesis. FUS = pulse-treated samples (two representative experiments given), P(+) = vacuolated protoplasts, P(−) = evacuolated protoplasts. (In the reference experiment (1 *g*), the fused suspensions were lost due to infection. Co-cultured parental protoplasts were substituted as reference material.)

	T (numbers)	D (%)	G (%)	M (%)
Samples exposed to microgravitation				
P(+), 1 *g* reference	37	3	3	94
P(−), 1 *g* reference	57	2	7	91
P(−), microgravity	74	0	0	100
FUS, microgravity	215	25	15	60
FUS, microgravity	176	3	25	72
Controls (1 *g*)				
P(+)	86	10	21	69
P(−)	129	6	10	84
FUS P(+)/P(−)	92	4	16	80
FUS P(+)/P(−)	89	7	24	69

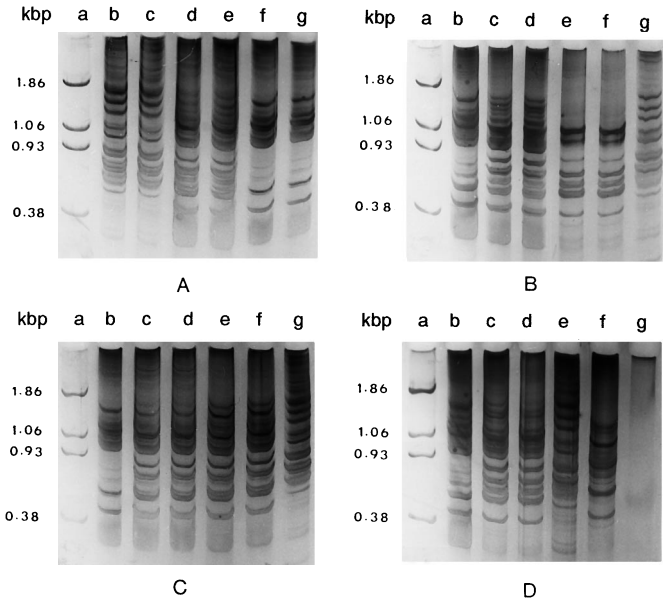


Fig. 4A–D. Analysis of DNA from *Nicotiana* species and hybrids by RAPD-PCR using primer UBC 344, separated in 8% polyacrylamide and stained with silver. The RAPD-PCR reactions were run over 40 cycles using 10–15 ng genomic DNA. **A** *Lane a*, molecular-weight marker (pBR322 DNA cut with Mva I); *lanes b–c*, *N. rustica; lanes d–e*, hybrid 3; *lanes f–g*, *N. tabacum* 'Xanthi'. **B** *Lane a*, molecular-weight marker (pBR322/Mva I); *lane b*, *N. tabacum* 'Xanthi'; *lanes c–d*, hybrid 9; *lanes e–f*, hybrid 13; *lane g*, N. *rustica*. **C** *Lane a*, molecular-weight marker (pBR322/Mva I); *lane b*, N. *tabacum* 'Xanthi'; *lanes c–d*, hybrid 29; *lanes e–f*, hybrid 15; *lane g*, *N. rustica*. **D** *Lane a*, molecular-weight marker (pBR322/Mva I); *lane b*, *N. tabacum,* 'Xanthi'; *lanes c–d*, hybrid 50; *lane e*, *N. rustica; lane f*, *Phaseolus P. vulgaris* (for comparison); *lane g*, negative control (all PCR components present except genomic DNA). (Data taken from De Filippis et al. 1996)

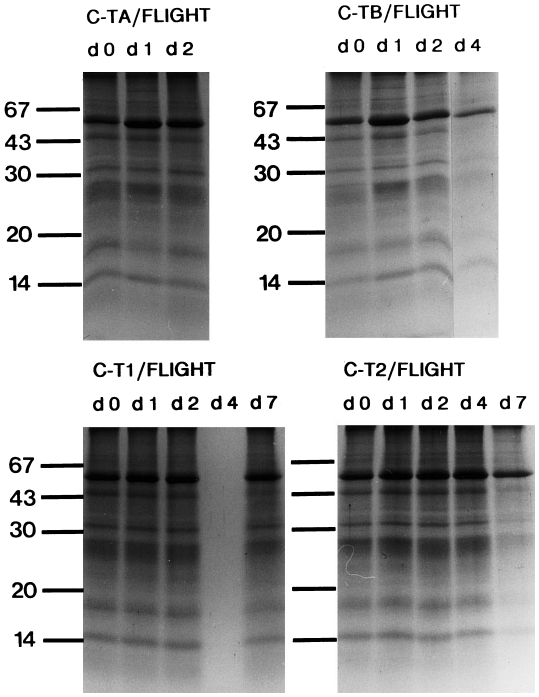


Fig. 5. Total protein pattern from *N. tabacum* after SDS-PAGE (flight samples; *top*, 1-*g* centrifuge; *bottom*, *μ-g* samples; *left*, vacuolated and *right*, evacuolated protoplasts). Electrophoresis was carried out under denaturing conditions. The numbers give the approximate molecular weights in kDa. d0–d7, days of incubation under microgravity; *C-TA/B/1/2,* different incubation chambers

control experiments, changes due to culture age started to appear between days 6 and 10; the duration of the spacelab experiment was thus too short to verify this effect: the latest microgravity samples were taken at day 7. Up to this length of cultivation, a major impact of the different growth conditions on the protein pattern could not be detected.

Immunoblotting of cytoskeletal proteins. Among the proteins which might be expected to respond specifically

Table 3. Morphological features and percentage of RAPD-PCR bands inherited from parental cells for tobacco somatic hybrids. Somatic hybrids were produced as a result of fusion between *N. tabacum* 'Xanthi' and *N. rustica,* and morphology assessed visually over a year in tissue culture. The RAPD-PCR summary is based on the banding patterns produced as a result of using four different primers which generated between 44 and 64 bands per sample. With reference to the molecular weights of the RAPD bands in the two parents, the percentage of bands derived from each of the two parents was calculated for each somatic hybrid. (Data taken from De Filippis et al. 1996)

Hybrid	Morphological description	RADP-PCR banding
3	Leaves asymmetrical and morphology of this hybrid closer to *N. rustica* than *N. tabacum* 'Xanthi'; good growth and root formation	Most bands inherited from *N. rustica* (68%), but some present were derived from *N. tabacum* 'Xanthi' (25%)
9	Leaves narrow and elongated; morphologically closer to *N. tabacum* 'Xanthi' than *N. rustica,* with vigorous growth, good roots	Most bands inherited from *N. tabacum* 'Xanthi' (68%) but a number were clearly derived from *N. rustica* (22%)
13	Leaves mostly vitrified, the few leaves not vitrified but present had morphology very similar to *N. rustica;* long petioles and no roots	A high proportion of bands were inherited from *N. rustica* (81%), and a low number were derived from *N. tabacum* 'Xanthi' (7%)
15	Leaves were also vitrified, no roots and small leaves; some normal leaf development was present and these leaves were similar to hybrid 3	Bands were inherited from both parents, roughly shared; 47% were from *N. rustica* and 42% came from *N. tabacum* 'Xanthi'
29	Leaves vitrified but some normal development was apparent, all were small and no roots present; leaves asymmetrical and similar to hybrid 15	Bands were derived from both parents and shared with 43% originating from *N. tabacum* 'Xanthi', 49% from *N. rustica*
50	Leaf morphology was very close to the shape of the parent *N. rustica,* vigorous growth, good rooting ability and dark green	Bands were mostly inherited from *N. rustica* (80%), but some bands were derived from *N. tabacum* 'Xanthi' (12%)

to changes in gravity are the components of the cytoskeleton. The acto-myosin system is thought to be involved in gravity sensing (Sievers et al. 1991), cytoplasmic streaming and organelle translocation (Staiger and Schliwa 1987). Microtubuli form a cortical network and seem to determine the direction of cellulose fibril deposition during cell wall formation (Lloyd et al. 1980). They also play a major role in cell division as constituents of the spindle apparatus and by determining the division plane (Flanders et al. 1990). In vitro, their self-assembly has been shown to be sensitive to gravity fields (Tabony and Job 1992). We thus screened total protein extracts for the cytoskeletal proteins tubulin (microtubules), and actin (microfilaments) by immunoblotting.

Polyclonal antibodies against homologous animal proteins cross-reacted with tobacco antigens and could be followed throughout the regeneration stages (Fig. 6). Pherograms were analyzed to determine molecular weights and to compare the relative intensities of bands (not shown). Actin is known to have a molecular weight of 42 kDa. One of the bands seen in the immunoblots (Fig. 6) was located at the corresponding position, and most likely represents tobacco actin. The intensity of this band as well as the cross-reaction at 30/32 kDa seemed

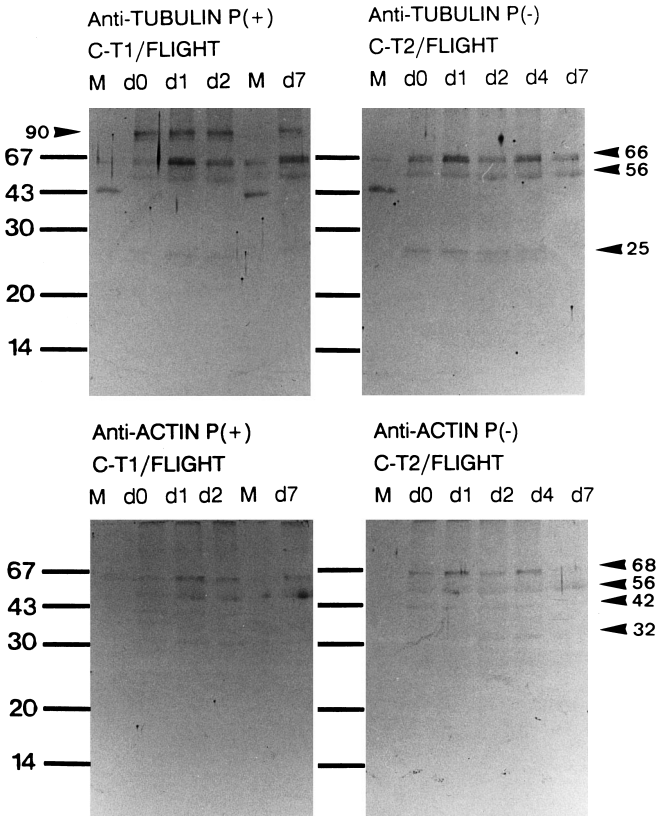


Fig. 6. Immunoblots from μ-*g* samples of vacuolated and evacuolated *N. tabacum* protoplasts with polyclonal tubulin and actin antibodies. The numbers give the approximate molecular weights in kDa. *Lane M*, marker proteins. (The marker protein cross-reacting with anti-tubulin serum is ovalbumin, from chicken eggs, which is recognized because the original antigen was isolated from chicken embryos.) Other abbreviations as in Fig. 5

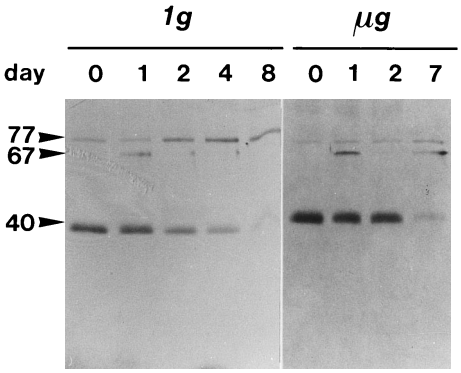


Fig. 7. Immunostaining for PAL from regenerating (vacuolated) *N. tabacum* protoplasts. The culture age (d) is indicated on top of the lanes; molecular weights are given in kDa. The amount of PAL protein increases with culture age for both reference (1 *g*) and microgravity samples

to increase with culture age. Differences in gravitation fields did not affect the actin pattern. The appearence of additional bands is common for polyclonal antisera. They may be caused by contaminations in the antigen used to raise the serum, by actin-related structures, or by degradation products of actin.

The molecular weight of tubulin is around 56 kDa. Again, a protein of this size was detected in the tobacco extracts along with some other bands (Fig. 6). The pattern was stable throughout the culture period and was not influenced by the experimental conditions. If microgravity does affect the composition of these cytoskeleton constituents, then the effects are too small to be detected by the blotting method used.

Immunoblotting of osmotin and phenylalanine ammonium-lyase (*PAL*). In addition to cytoskeletal proteins we blotted for a stress-indicating pathogenesis-related protein, osmotin, and for PAL, a marker enzyme for an initial step into secondary metabolism. With increasing culture age we found both an increase in density of presumptive PAL bands (77 kDa, Fig. 7; for discussion see Hoffmann et al. 1996) and of an osmotin band (23 kDa; Fig. 8). The presence of the latter can be taken to indicate a regular stress response of protoplasts (Grosset et al. 1990). Again, no gravity-related effects could be detected.

Pool sizes of metabolites. Metabolic flux control is exerted both by the amount or activation state of rate-limiting enzymes and by the pool sizes of regulatory metabolites for a given pathway (Stitt 1994). Whereas the in vivo activation state of an enzyme is difficult to assay, especially under limited experimental possibilities, pool sizes of metabolites can easily be conserved using appropriate methods for metabolic quenching. We thus used the latter approach.

An alteration in the balance between energy-consuming (anabolic) and energy-producing (catabolic) pathways can cause altered ratios of compounds such as adenine and pyridine nucleotides which constitute

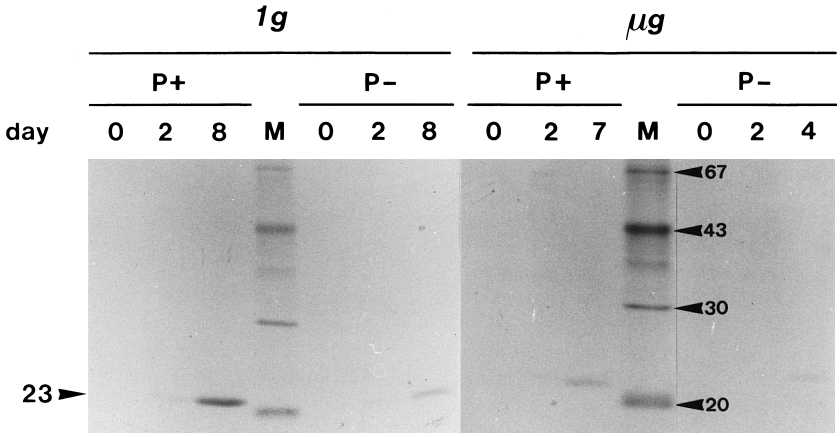


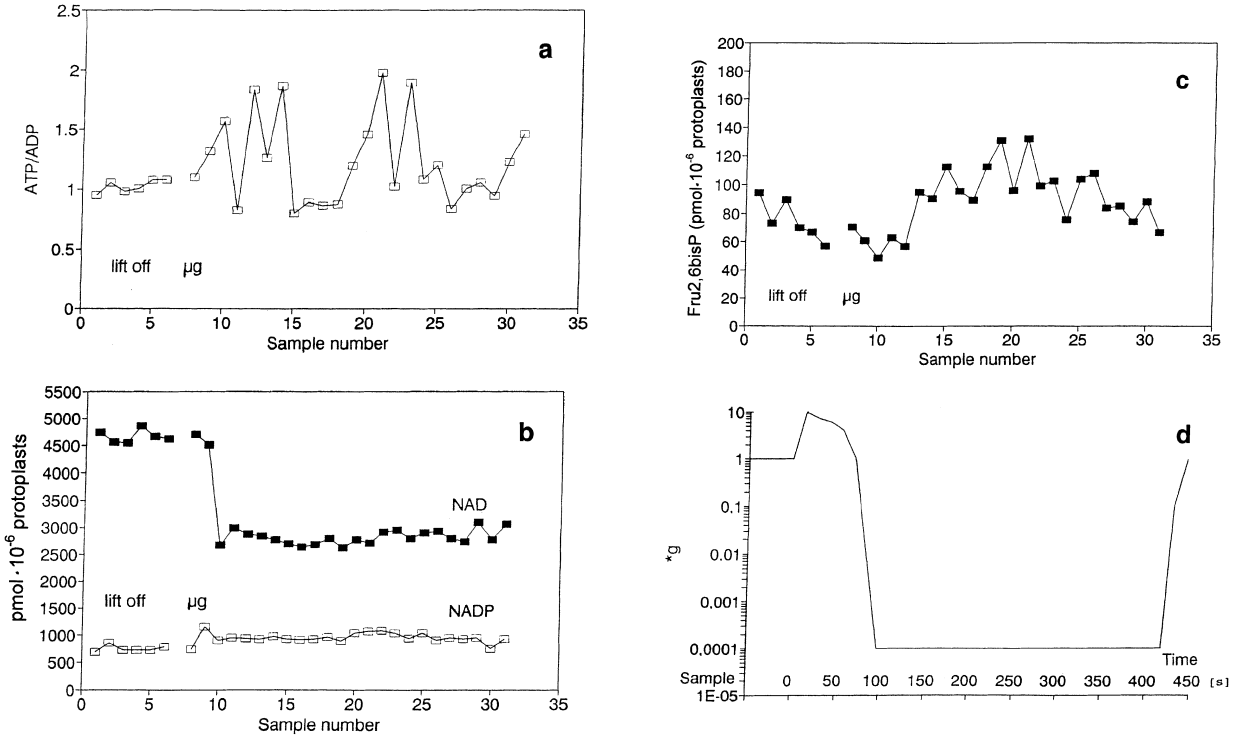
Fig. 8. Immunostaining for osmotin from regenerating *N. tabacum* protoplasts. The culture age (d) is indicated on top of the lanes; molecular weights are given in kDa. *Lane M*, biotinylated marker proteins visualized by an avidine alkaline-phosphatase conjugate. P(+)/P(−), vacuolated/evacuolated tobacco protoplasts as starting material for culture. Osmotin protein is detectable from 4 d of culture onwards, independent of treatment

important cofactors of these pathways. The ratio of ATP/ADP, for example, has been shown to increase when cells are under stress and has thus been taken as a measure for enhanced anabolic activity (Atkinson 1977). As ATP formation is coupled to respiratory oxidation of NADH, the redox ratio of the NAD system (NADH/NAD) is another measure which can characterize the overall metabolic stage of a cellular system.

In animal as well as non-illuminated plant cells energy for anabolic metabolism stems from the oxidation of carbohydrates via glycolysis and connected pathways. Most steps of glycolysis are reversible and thus also allow the regeneration of carbohydrates. This path is called gluconeogenesis. A key step in gluconeogenesis, the formation of fructose 6-phosphate from fructose-1,6-phosphate (Fru 1,6 bisp), is inhibited by Fru 2,6 bisp in micromolar concentrations, while in parallel, a plant-specific kinase, catalysing the interconversion of fructose 6-phosphate and Fru 1,6 bisp with a preponderance (4:1) of the glycolytic direction is highly activated (Stitt 1990). Even a slight rise in the level of Fru 2,6 bisp strongly inhibits gluconeogenesis and is thus a sensitive indicator for the balance between glycolysis and gluconeogenesis.

Short-term changes in reduced gravity. Pool sizes of ATP, ADP, NAD, and Fru 2,6 bisp were determined from suspensions of tobacco mesophyll protoplasts (*N. tabacum*) during parabolic flights offering 10 min of reduced gravity (Fig. 9). Samples were quenched by an automated device at 19-s intervals by injecting either acid or base (final concentration 0.1 M HCl: oxidized

Fig. 9a–d. Alterations in metabolite concentrations and ratios of *N. tabacum* mesophyll protoplasts upon transition to microgravity during sounding-rocket flights. **a** Ratio of ATP/ADP; **b** amount of NAD; **c** amount of fructose-2,6-bisphosphate (F26BP); **d** profile of *g*-forces. (Data compiled from different TEXUS experiments)



pyridine nucleotides; 0.02 N NaOH: Fru 2,6 bisp; 10% $HClO_4$: adenylates) into the respective protoplast suspension (Hoffmann et al. 1995a). Upon transition to microgravity we found increased fluctuations in the ratio of ATP/ADP, decreased levels of NAD, and transiently increased amounts of Fru 2,6 bisp. Obviously these data are very limited: the amount of NADH could not be determined in parallel because of hardware limitations, and although the samples were taken in triplicate, an independent repetition has not yet been possible. From reference experiments we know that a change in the total pool size of the NAD system (NADH + NAD) within 10 min of incubation is not likely in protoplast suspensions. It is thus reasonable to assume that a decrease in NAD should be accompanied by an increase in NADH.

Long-term changes in reduced gravity. Protoplast culture is characterized by a steady increase in the contents of ATP, NAD and Fru 2,6 bisp up to day 4 or 6. Highest amounts of these metabolites were detected when cells started to divide. With regard to adenine and pyridine nucleotides, comparable changes have been reported for cell-suspension cultures (Shimizu et al. 1977; Shimazaki et al. 1982). Cultures of evacuolated protoplasts show the same behaviour, but are usually delayed in comparison to normal mesophyll protoplasts. This pattern was not affected by microgravity.

Adenine nucleotides. Changes of pool sizes of ATP and ADP for cells grown on the ground and in the 1-*g* reference centrifuge (spacelab) were quite similar. In both cases the ATP content increased from about 1.8 (day 0) to 3.7 nmol · (mg protein)$^{-1}$ (day 6). The ATP content of the culture in microgravity was lower, starting at 1.4 nmol · (mg protein)$^{-1}$ with a slower increase up to 3.5 nmol · (mg protein)$^{-1}$ at day 7 (for direct comparison: 3.0 nmol · (mg protein)$^{-1}$ at day 6). This converts to a reduction in the ATP content of ca. 20%, which is also expressed by the ATP/ADP ratio (Fig. 10). This ratio from cultures in microgravity was consistently below that of 1-*g* controls.

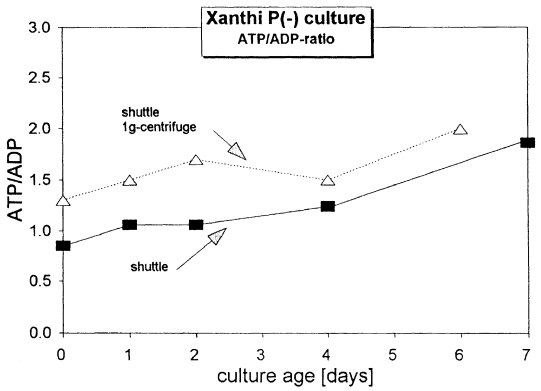


Fig. 10. Ratios of ATP/ADP of regenerating *N. tabacum* mesophyll protoplasts. Example for a culture which was started with evacuolated protoplasts

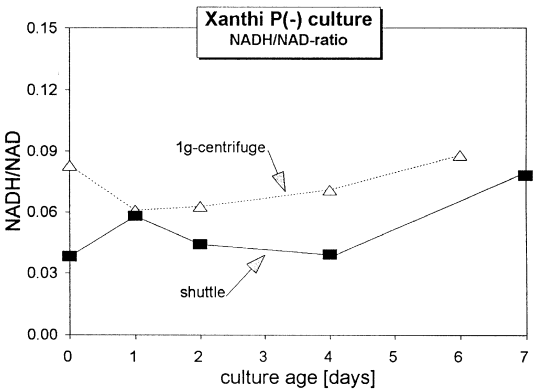


Fig. 11. Ratios of NADH/NAD (redox charge) of regenerating *N. tabacum* mesophyll protoplasts. Example for a culture which was started with evacuolated protoplasts

Pyridine nucleotides. The most abundant pyridine nucleotide in tobacco protoplasts is NAD. During cultivation the content of NAD (and its counterpart NADH) increased in parallel with the start of cell wall formation and cell expansion. In 1-*g* cultures, the content of NAD + NADH increased from 3 to 5 nmol · (mg protein)$^{-1}$ within 6 d. This increase was slower and smaller in cultures under microgravity (4 nmol · (mg protein)$^{-1}$ after 7 d (i.e. again a 20% reduction). More important, and similar to the adenylate system, the NADH/NAD ratio was generally lower than in the controls (Fig. 11). Contents of the components of the NADP system (NADPH, NADP) were much lower (total pool size well below 1 nmol · (mg protein)$^{-1}$) and exhibited no development-related changes (not shown).

Fructose-2,6-bisphosphate. Figure 12 shows the change of Fru 2,6 bisp content during protoplast cultivation. Ground cultures and those in the reference centrifuge in the spacelab behaved in the same way. Within the first 2 d, the Fru 2,6 bisp concentration doubled from 5 to 10–12 pmol · (mg protein)$^{-1}$, and then stayed at this level until the end of the investigation period. In samples from cultures, in microgravity, Fru 2,6 bisp was

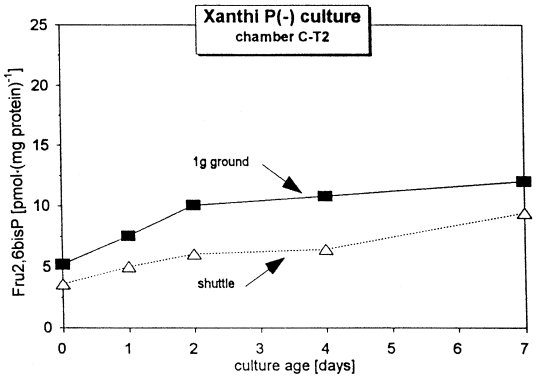


Fig. 12. Fructose-2,6-bisphosphate content in regenerating *N. tabacum* mesophyll protoplasts. Example for a culture which was started with evacuolated protoplasts. 1 g_{ground} and 1 $g_{(reference\ centrifuge)}$ were identical

decreased (3–9 pmol · (mg protein)$^{-1}$, or 60–75% of the content of 1-*g* controls). This observation could indicate a decreased preference for glycolysis.

Discussion

In this contribution we summarize data on the first successful regeneration of plant protoplasts maintained in culture under microgravity for about 10 d together with data for an attempted metabolic analysis. Our data on protoplast culture and their subsequent ability for regeneration give no indication of microgravity-induced alterations with respect to 1-*g* controls. This is in contrast to another trial under microgravity with plant cell protoplasts (Rasmussen et al. 1992). These authors reported for rapeseed a retardation of development under microgravity. According to their interpretation, the lack of cell-to-cell contact between the free-floating cells was the reason why only a few, and small, cell aggregates were formed during a 14-d period in orbit. The protein content of these cultures (μg · (μl packed cell volume)$^{-1}$) was reduced by 71%, and distinct protein bands were missing in the electrophoresis pattern (SDS-PAGE and native isoelectric focussing) of flight samples. In our experiment, differences either in the protein content or in the pattern of SDS-electrophoresis were not visible. This is consistent with the apparently regular development of the cultures. It is thus quite possible that experiment culture conditions were not optimal in the rapeseed.

There is experimental evidence that the cytoskeleton is involved in the gravity signal perception/transduction mechanism (Volkmann and Sievers 1992a, b; Guikema et al. 1993; Hilaire et al. 1995), and recent experiments show that a microtubular network also develops under microgravity (Hilaire et al. 1995). Our data indicate that the cytoskeletal components, actin and tubulin, were obviously not altered, at least not so much that we were able to detect changes via Western blotting. This does not exclude that, with the same amount of monomers present in the cell, the degree of polymerization of the filaments was different.

Although the microtubular system by itself is possibly not affected, its energy turnover could well be, if one assumes that the positioning of cell organelles under microgravity needs less metabolic energy owing to missing sedimentation. This is the reason why we tried to characterize cell metabolism, especially energy metabolism. Most interestingly, changes in pool sizes with respect to controls of Fru 2,6 bisp and of the redox and energy status were opposite during short-term (10 min) and long-term (up to 7 days) exposure to microgravity. One could speculate and summarize the short-term effects (increased Fru 2,6 bisp pool and ratios of NADH/NAD and, possibly, of ATP/ADP) as being indicative of a stress response upon transition from 1 *g* to hypergravity (acceleration) and then to microgravity, as tissues under, e.g., environmental stress, respond in the same way (see above). The lowered ratios of NADH/NAD (redox charge) and of ATP/ADP, as well as the decreased amount of Fru 2,6 bisp, during long-term exposure should therefore reflect the opposite of metabolic stress. Thus, one could assume a metabolic relaxation in cell cultures maintained under microgravity.

We do not, however, know to what extent cell metabolism during culture is affected by limited nutrient or gas diffusion which should be different between microgravity and 1 *g* (reference centrifuge) samples (the former were only moved for sampling). Nutrient supply, at least, should not have been limiting, as the ratio of cell volume to total culture volume was kept below 2/1000. This should also have prevented local oxygen depletion. Future experiments with cell cultures should, however, include suitable sensors. Obviously, such limitations in cell activity, although possible, did not apply as rates of cell division and of callus formation were not different between microgravity and 1-*g* samples.

Our investigation into the impact of gravity on energy metabolism of plant protoplasts is so far a unique experiment, but the results of some experiments with other organisms show similar trends. Bacteria, for example, seem to grow faster under microgravity (Mennigmann and Lange 1986; Mennigmann and Heise 1995). This could result from a surplus of metabolic energy that is not subject to intracellular down-regulation. Our plant cells did not grow faster under microgravity but their metabolic turnover within steps of primary (glycolysis) and intermediary metabolism (energy status) could have been slowed down. This could support secondary pathways which are normally limited as long as the needs for growth and maintenance are not covered. In order to address this possibility, we used some of our samples to assay and quantify a key enzyme of secondary metabolism, PAL. With respect to variation within individual samples, however, we have not yet been able to verify this assumption.

We are well aware about the large discrepancy between the amount of sound data and assumptions based thereupon. However, we think it should be worthwhile persuing the hypotheses presented in future experiments.

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