Effects of Microgravitation on Electrofusion of Plant Cell Protoplasts

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ABSTRACT

Electrofusion of evacuolated with vacuolated mesophyll protoplasts of Nicotiana tabacum was performed as part of the German Sounding Rocket Program (TEXUS 17, 1988). The results indicate a significant increase not only in the yield of 1:1 hybrids, but also in homo- and multifuusion products. Hybrids obtained under microgravity have been shown to be viable to a higher degree with respect to their ability for light-dependent O2-evolution (independent of other substrates than bicarbonate). This finding is of interest for fusion experiments were only limited numbers of fusion partners are available (e.g. protoplasts from embryogenic tissues) or where fusion yields are extremely low under 1 x gravity (e.g. protoplasts of different specific density).

Fusion of plant cell protoplasts is a prerequisite for somatic hybridization and the exchange or recombination of organelles between incompatible species. Early attempts to induce protoplast fusion successfully employed high pH and calcium (10), polyethylene glycol (2, 10), or dextran (9). However, a drawback these methods have in common is that chemical fusogens are eventually toxic and unreliable, and that the process of fusion cannot be controlled.

Another approach, fusion of protoplasts by the reversible electric breakdown of their plasma membranes (43), has been shown to be an efficient alternative to conventional methods. Furthermore, if selected hybrid cells are to be formed, electrofusion is the method of choice.

In spite of the considerable potential offered by this field pulse technique, there are some inherent physical and technical, and biochemical factors which reduce the theoretically achievable yield of viable hybrids under terrestrial gravitation (12). Optimum yields of hybrids are only obtained, if the aligned cells do not change their position for a limited time after pulse application. Only then, persistent membrane continuities can develop. Under terrestrial conditions, however, gravitational and convectional forces severely interact. For example, cell nuclei or organelles, such as chloroplasts or amyloplasts, are not centered and thus cell rotation occurs. It is thus imperative to apply and to perpetuate field strengths higher and longer than necessary in order to keep the fusion partners aligned. In addition to suboptimal fusion yields, this can cause a decrease in hybrid viability.

With electrofusion of plant protoplasts there are additional problems. Mature plant cells possess a large lytic compartment, the vacuole. This compartment is separated from the center of metabolic activity, the cytosol, by the tonoplast membrane. Tonoplast and outer cell membrane (plasmalemma) can come into close spacial contact. Thus pulse application can lead to a perforation of both membranes (44). While perforation of the outer membrane is the basic step for fusion of aligned protoplasts, poration-induced leaching of solutes from the vacuole will lead to a decreased cell viability and should thus be avoided. Fusion of evacuolated plant protoplasts can overcome this problem. However, regeneration of plant protoplasts, starting with cell wall formation, requires a lytic compartment and has shown to be considerably slower with evacuolated protoplasts (1) (B Naton et al., unpublished data). Formation of heterospecific hybrids from vacuolated and evacuolated protoplasts appears thus to be ideal. However, owing to the considerably different specific densities of both types of protoplasts (tobacco mesophyll protoplasts: vacuolated, 1.05; evaculated 1.10) it is not possible to have both suspended in the same medium; while one type is suspended, the other will either floatate or sediment. In consequence, it is very difficult to establish close membrane contact between the fusion partners and fusion events are extremely rare. From material sciences we know that under microgravitation interferences resulting from convection and gravity do not exist. Therefore, electrofusion under microgravity should circumvent these problems (20). Indeed, Zimmermann et al. (19) have shown that electrofusion of yeast protoplasts under 5-min microgravity resulted in a significant increase in hybrids. More recently, these authors observed that under similar conditions the fusion yield of mammalian cells increased by a factor of 10, while the number of viable hybrids was about twice as high as under terrestrial gravity (U Zimmermann, P Kleinhans, R Schnettler, unpublished data; see also Ref. 20).

In this contribution we report on a fusion experiment which was performed as part of an unmanned German Sounding Rocket Program (TEXUS) (3). The results indicate a significant increase of viable heterospecific 1:1 hybrids after alignment and electrofusion under microgravity.

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**ELECTROFUSION UNDER MICROGRAVITY**

**Figure 1.** Electrofusion module TEM 06-5 assembled and ready for integration into the payload unit. The lateral window allows for late access: fusion chamber, storage units for protoplasts and mixing device (peristaltic pump) are mounted on a separate platform and can be added to the module about 2 h before takeoff.

**Figure 2.** Detailed view of the fusion chamber.

**Figure 3.** Schematic diagramm of the experimental system.

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**FUNCTION SCHEME**

1 = FUSION CHAMBER  
2 = ELECTRODE  
3 = FUSION MEDIUM (SORBIT)  
4 = MEMBRAN  
5 = STORAGE UNIT  
6 = MIXING CHAMBER  
7 = PROTOPLASTS  
8 = TUBES  
9 = PERESTALTIC PUMP  
10 = PELTIERELEMENTS
MATERIALS AND METHODS

Plant Material

Mesophyll protoplasts from Nicotiana tabacum (cv. Samsun) were isolated from 4 to 5 week old plants by flotation of leaf pieces (lower epidermis abraded) on 0.6 M sorbitol, containing 1 mM CaCl₂, 5 mM Mes-KOH (pH 5.6), 10 mM ascorbic acid, 0.5% (w/v) BSA, 2% cellulase “Onozuka R-10,” and 0.5% (w/v) Macerozyme R-10 (both Serva, Heidelberg, FRG) (6). Evacuation was according to Griesbach and Sink (4) with the following modifications. Purified protoplasts (0.5 ml, about 1.5 - 10⁶) were layered on top of 3.7 ml Percoll, to which were added (all undissolved) sorbitol (0.5 M), CaCl₂ (100 mM), and Mops (5 mM, pH adjusted to 7.0). Centrifugation (4.4 mL polyallomer tubes) was at 117,000g and for 40 min (23°C; swing out rotor). This resulted in several layers of which the uppermost ones (pure vacuoles, plasmalemma surrounded vacuoles, cytoplasmic aggregates) were discarded. The layers containing evacuated protoplasts were carefully removed by suction and washed twice with 0.5 M sorbitol.

Vacuolated protoplasts (P⁺; Fig. 4a) were stored in an isotonic solution containing 0.25 M sorbitol, 0.25 M sucrose, 1 mM CaCl₂, 5 mM Mes-KOH (pH 6). For storage of evacuolated protoplasts (P⁻; Fig. 4b), sorbitol was substituted by 0.25 M raffinose. For increased stability a volume/particle ratio of 2 ml per 1 ⁴³ protoplasts was found suitable. About 2 h before starting the experiment protoplast density was brought to about 4 ⁴⁻⁶/mL.

Hardware Design for Radio-Controlled Electrofusion under Microgravitation and Data Transmission

The module used for the experiment (Figs. 1 and 2, ERNO, Bremen, FRG) consisted of the fusion chamber, two storage units for evacuated and sucrose protoplasts respectively, a reservoir for fusion medium, a peristaltic pump, and facilities for optical control of the interelectrode space of the fusion chamber (Fig. 3). Connections between the different containers were made from silicon tubings (inner diameter: 1 mm). In order to compensate for changes in volume during resuspension of the protoplasts and filling of the fusion chamber all storage units (containers for protoplasts and fusion medium) were closed on one end by flexible membranes (Freudenberg, Weinheim, FRG).

In order to introduce significant numbers of protoplasts into the fusion process a meander-shaped fusion chamber was constructed (Fig. 2). The electrodes (stainless steel) were fabricated by spark-erosion (Witte, Barskamp, FRG). Owing to an electrode distance of 1.2 mm, a gap height of 8 mm, and a total length of the interelectrode space of 520 mm, about 5 mL of protoplast suspension could be exposed to the necessary electric field. For optical control of the interelectrode space, the electrode housing was made from plexiglass. The fusion chamber was mounted on a moveable platform. By means of a step motor it was thus possible to adjust the position of the chamber in the direction of the light path of the microscope optics (perpendicular to the chamber surface; total magnification 100-200×).

The complete experimental unit (except optics) was mounted on a separate platform which could be introduced into the module through a lateral window. This allowed for free access of our samples to the assembled payload about 2 h before takeoff. Thermal control of the cell containers was achieved by Pelitier elements. This was necessary to maintain the protoplast suspensions (a) at 6 to 8°C until about 10 min before takeoff, (b) at 20°C during the electrofusion experiment.

Conditions for Electrofusion of Vacuolated and Evacuolated Tobacco Mesophyll Protoplasts

About 80 s after launch the peristaltic pump was activated and both types of protoplasts (0.4 mL each) were resuspended by dilution in their storage units to 8 mL with fusion medium (0.5 M sorbitol). By changing the pumping direction the protoplasts were withdrawn from their respective chambers under continuous mixing in a ratio 1:1. Times for mixing and filling were 15 to 20 s each (20°C). After ending the filling procedure, protoplasts were collected by a weak alternating field (2 MHz; 160 V/cm, peak to peak) under optical control and as soon as short protoplast chains were visible the fusion pulse was applied (0.9 kV/cm; 50 μs). Part of the processing of the sample was in an automatic mode but critical steps (pump activation, flow direction and pulse application) could be directed by telecontrol.

Evaluation of Fusion Yield and Physiological Integrity of Hybrids

Within 1 h after pulse application (reference test 1g as well as experiment under microgravitation) the protoplast suspension was washed out of the fusion chamber by about 50 mL of 0.5 M sorbitol, containing 1 mM CaCl₂ and 5 mM Mes-KOH (pH 6). Preeparation of particle populations of differ-

Figure 4. Vacuolate (a) and evacuate (b) mesophyll protoplasts from Nicotiana tabacum (cv. Samsun).

Figure 5. Particle distribution in a mixture of vacuolated and evacuated tobacco mesophyll protoplasts in a 1g-reference experiment 10 s after filling of the fusion chamber. Most of the evacuated protoplasts sedimented to the lower electrode surface.

Figure 6. Same view as in Figure 5, but under microgravitation. In contrast to the reference experiment (1g, Fig. 5), protoplast distribution is rather homogeneous. The photographs are taken from video recordings and show the electrode gap.

Figure 7. Fused protoplast suspension after recovery and return of the payload to the launch center (within 1 h after takeoff). Hybrids are indicated by arrows.

Figure 8. Protoplast fraction enriched with vacuolate x evacuate hybrids by centrifugation on a self-generated Percoll gradient.

Figure 9. Bacterial assay (Pseudomonas aeruginosa) of light-dependent O₂-evolution of a viable hybrid protoplast. a, Protoplast after an illumination period of 5 min. Note the increased density of bacteria on that surface area with the high chloroplast number (dark spot; evacuate part of the hybrid). b, Same protoplast after dark treatment (flash light photomicrographs).
ent densities was by sedimentation for 1 h under 1g (6°C). This procedure was repeated with the remaining supernatant. For enrichment of heterospecific 1:1 fusion products the pellet obtained by this second sedimentation step (1 h as above) was combined with the pellet resulting from centrifugation of the second supernatant (10 min, about 50g) and layered on top of a preformed Percoll gradient (15).

The yield of heterospecific hybrids (vacuolated × evacuolated protoplast) was determined microscopically by counting aliquots of the initial washout on a hemocytometer (Fuchs-Rosenthal). Physiological integrity of the hybrid protoplasts was tested by their individual ability to evolve O₂ in the light. The assay which employs aerotactic bacteria (Pseudomonas aeruginosa) is described in detail elsewhere (6).

All steps of preparation and handling of the samples were performed at ESRANGE (Swedish Space Corporation, Kiruna, Sweden).

RESULTS AND DISCUSSION

Electrofusion of a Mixture of Vacuolated and Evacuolated Tobacco Mesophyll Protoplasts under Terrestrial Gravitation (1g)

The experimental set up as described above was used for control experiments under terrestrial gravitation. In order to obtain optimum fusion rates the fusion chamber was filled under different spacial orientations. Best results were obtained with the long side of the meander perpendicular to the gravity vector (see Fig. 2). Independent of the chamber orientation, the mixing and filling procedure resulted in a very homogeneous distribution of both vacuolated and evacuated protoplasts over the total length of the interelectrode space. Immediately after filling, however, a rapid separation of both particle populations started, i.e. while the vacuolated protoplasts remained suspended the evacuated ones sedimented. This led to a very inhomogeneous distribution of both types of protoplasts within seconds (Fig. 5) and could only be slightly retarded by the immediate application of the alternating electric field (positive dielectrophoresis). Thus, primarily homospecific pair and chain formation occurred (P⁺ × P⁺, P⁻ × P⁻). As a consequence, electric pulse induced fusion created only small numbers of P⁺ × P⁻ hybrids (between 0.7 and 1.0% of the total protoplast population).

Electrofusion under Microgravitation

Radiocontrolled mixing and filling of the fusion chamber under microgravitation caused a homogeneous distribution of both protoplast preparations which was completely stable when the filling step was ended (Fig. 6). The only visible movement of the protoplasts relative to each other occurred when the collecting A.C. field was applied. After a 20-s A.C. field protoplast pairs started to grow into chains of several protoplasts and thus the alternating field was switched off. By setting a square pulse (0.9 kV/cm, 50 μs) fusion was initiated, and again no particle movement was visible. This is in significant contrast to fusion under 1g; here, sedimentation and convective forces induce movement of the fusion partners relative to each other which eventually leads to a breakdown of newly formed membrane continuities. Thus, typically a post-fusion weak alternating field has to be applied in order to prevent a separation of the fusion partners. This, however, affects hybrid viability (our unpublished observations). Our experimental data show that this is not necessary under weightlessness.

Microscope analysis of the exposed protoplast suspension after retrieval (less than 1 h after fusion) showed a significantly increased portion of hybrid cells, of both homo- and heterospecific nature (Fig. 7). Evaluation of about 1000 protoplasts (vacuolated and evacuated) yielded about 120 clearly distinguishable 1:1 hybrids, i.e. about 12% of all cells submitted to the fusion procedure. This is about 10 to 15 times more compared to fusion under terrestrial conditions and, in real numbers, about 0.5·10⁶ heterospecific 1:1 hybrids out of 4·10⁶ protoplasts introduced into the fusion chamber. This yield is sufficiently high to be used for biochemical analysis, such as protein analysis or characterization of the metabolic state (e.g. Ref. 5).

Similarly, the yield of homofusion and multifusion products (hybrids formed from either vacuolated or evacuated protoplasts) was also increased, although to a lesser extent. In this case the increase in yield should not be a matter of missing differential sedimentation velocity, as, owing to the isolation procedure (purification on a density gradient) there should be no larger difference in specific density within such a protoplast population. Instead we believe that it is the lack of convectional movement which increased the yield. As mentioned above, a weak alternating electric field has to be applied after pulse application in order to prevent membrane bridges from breaking down. In a solution without any conductivity this would not cause thermal problems. There is, however, always some leakage of solutes from decomposing protoplasts, which increases conductivity. Thus some current will pass the protoplast suspension and cause local increases in temperature. This, in consequence, causes convectional movement and, finally, a decreased number of hybrid cells.

As far as hybrids from protoplasts with comparable specific density (P⁺ × P⁺, P⁻ × P⁻) could still be identified without doubt (about 2-4 h after microgravity fusion), the yield with respect to the total number of vacuolated protoplasts was about twice compared to terrestrial conditions (10.5% instead of 4.5%). This is, however, an underestimate. Owing to the more or less rapid reorganization of a vacuolated hybrid from tobacco mesophyll protoplasts we were possibly only able to identify a fraction of the totally formed hybrids. Under terrestrial conditions only up to 50% of the hybrids recognizable within minutes after electrofusion can be identified by recounting 2 h later (storage at 4°C). As the microgravity-exposed samples were kept under comparable conditions, the real increase in yield could be considerably higher. Such an evaluation is not a problem with hybrids formed from vacuolated × evacuated protoplasts. Here the viscosity of cytoplasm of the evacuated partner is that high that complete mixing takes up to 2 d.

Centrifugation of the pulsed protoplast suspension on a preformed sigmoidal Percoll gradient (15) yielded a fraction enriched with up to 27% heterospecific 1:1 hybrids (Fig. 8). Hybrids from this fraction were assayed for physiological integrity by qualifying their individual ability for photosyn-
thetic oxygen evolution (6) (Fig. 9, a and b). This test system which employs aerotactic bacteria (*Pseudomonas aeruginosa*) indicated that nearly all hybrids (more than 90%) obtained by electrofusion under microgravitation were viable according to this standard. This is significantly more compared to terrestrial conditions (about 50–60%).

In summary, we have shown that electrofusion under conditions of weightlessness can be used to significantly increase not only the yield of hybrids from parental protoplasts with considerable differences in specific density but also in general as detrimental effects caused by particle sedimentation and convectional forces are excluded. This finding is of significance for fusion experiments were only limited numbers of fusion partners are available (e.g. protoplasts from embryogenic tissues) or certain animal cells (13, 18) or where fusion yields are extremely low under 1g (e.g. protoplasts of different specific density) (7, 8, 16).

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LITERATURE CITED