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FUSION OF *AVENA SATIVA* MESOPHYLL CELL PROTOPLASTS BY ELECTRICAL BREAKDOWN *

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Summary

Studies with the light microscope were carried out on mesophyll cell protoplasts of *Avena sativa* which had been made to undergo fusion by reversible electrical breakdown of the cell membrane. In order to establish close membrane contact between the cells, an important prerequisite for fusion, a method known as dielectrophoresis was used. In an inhomogeneous alternating electrical field the protoplasts adhere to the electrodes and to each other in the direction of the field lines. The cells which were thus brought into close contact with each other could be made to fuse by the application of a field pulse of high amplitude (about 750 V/cm) and short duration (20—50 μs). The field strength required for fusion exceeds the value necessary for the electrical breakdown of the cell membrane. Fusion took place within some minutes and led to a high yield of fused protoplasts. The fusion of cells being in the electric field occurred in a synchronous manner. In some of the fusion experiments part of the protoplasts of *A. sativa* were stained with neutral red. When these cells were fused with unstained protoplasts, the vacuoles from the different cells within the fused aggregate could be shown to remain separate for quite some time.

Membrane and cell-to-cell fusion are involved in several biological phenomena such as exo- and endocytosis, fertilisation and biogenesis of muscle fibers [1,2]. However, most membranes do not fuse under normal conditions, and

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clearly this is necessary in order to prevent uncontrolled fusion of tissue cells and also to ensure that the various membrane-bound organelles retain their structural identity, thus enabling different functional activities to be localised within specific compartments of the cell. Membrane fusion and cell-to-cell fusion can be initiated in vitro with the aid of viruses (e.g., inactivated Sendai virus) or chemical agents (poly(ethylene glycol), dimethyl sulfoxide and lipids [2—5]). In both cases, the plasma membranes of the cells to be fused are required to be in close juxtaposition. The major disadvantage shared by all fusion techniques is the extreme difficulty in achieving rapid and synchronised fusion [3] of a large number of cells. A high yield of two-celled fusion products is of particular interest because of its implications for somatic hybridisation [6].

In the experiments reported here, we demonstrate that fusion of mesophyll cell protoplasts of *Avena sativa* occurs as a result of electrical breakdown of the membranes of cells that are in close contact with each other. Contact between two or more cells was achieved by applying an inhomogeneous alternating electrical field of low intensity to the cell suspension. By simple physical laws, the cells are drawn into the region of high field intensity, and in the process they form chains with very close membrane contact [7—9].

Mesophyll cell protoplasts were obtained using the following procedure [10]. After sowing, *A. sativa* was kept in the dark for 5 days at 20°C. After about 2—4 days in a light-dark rhythm of 16 or 8 h the plants were used for the isolation of protoplasts. The leaves were divided into 1 mm wide strips which were left in a 4% cellulysin solution (Calbiochem, CA, U.S.A.) for 4 h at pH 5.3 (0.4 M mannitol, 1 mM $CaCl_2$). After filtration through a sieve with a 50 μm mesh, the pellet (centrifuged at 50 $\times g$ for 5 min) was resuspended in 5 ml of a solution containing 0.5 M sucrose and 1 mM $CaCl_2$. The suspension was covered with 2 ml of a 0.5 M mannitol solution (1 mM $CaCl_2$) and centrifuged for 10 min at 200 $\times g$. The protoplast fraction from the boundary layer was washed in an ion-free 0.5 M mannitol solution and immediately used for the experiments. The principle and the experimental set-up for the dielectrophoretic and fusion experiments have been described in detail elsewhere [9—13]. Briefly, a function generator (type 7404 P, supplied by the firm Toellner) is connected in parallel with a pulse generator (type 214 B, Hewlett-Packard) via a 50 Ω resistor and to the two electrodes in the dielectrophoretic chamber. The electrodes and the cell suspension surrounding them were observed under the microscope. The chamber had a volume of 1 ml, and the chamber wall with the two horizontally mounted electrodes was glued to a slide. The two electrodes consisted of two parallel cylindrical gold-plated brass rods with a diameter of 3 mm. The shape of the electrodes is such that a strongly inhomogeneous electrical field is produced. The minimum distance between the electrodes was 200 μm. When suspended mesophyll cell protoplasts of *A. sativa* are subjected to a non-uniform alternating electrical field between two electrodes, the cells are found to move towards the electrodes and to form chains in the process (Fig. 1). The length of these chains depends on the suspension density and on the frequency and duration of the applied field. This effect, which was first discovered by Crane and Pohl [11] in yeast, is known as dielectrophoresis. Dielectrophoresis is defined as the movement of matter in a non-

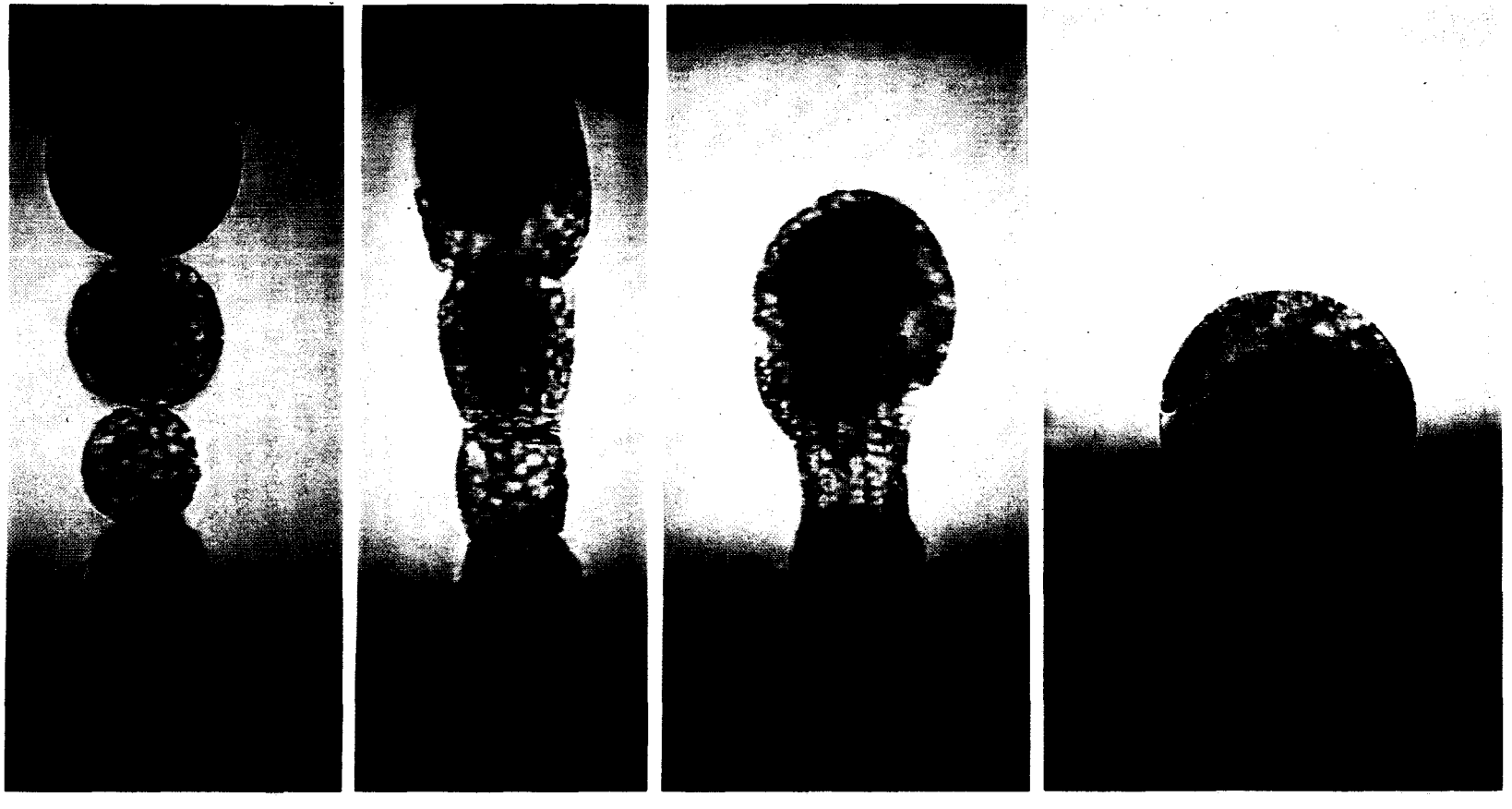

Fig. 1. Cell-to-cell fusion between mesophyll cell protoplasts of *Avena sativa* triggered by electrical breakdown of the membranes. The protoplasts (partly stained with neutral red) were suspended in a solution consisting of 0.5 M mannitol and collected at the electrodes by dielectrophoresis (sine wave, 500 kHz, 4 V amplitude). After reduction of the dielectrophoresis voltage to an amplitude of 2 V a single square pulse was applied (15 V, 20 μs). The photographs (from left to right) show the protoplast arrangement obtained by dielectrophoresis before application of the pulse and the cells during the time course of fusion (10 300 and 600 s after application of the pulse, respectively).

uniform electrical field, and is caused by polarisation effects. Electrophoresis of cells, caused by the negative charge on the cell surface, does not occur because the applied electrical field is alternating. When the electrical field is removed, the chains of cells disintegrate again because of Brownian motion and because the negatively charged cells repel each other. The formation of chains of *A. sativa* protoplasts is only reversible as long as the voltage, which is applied in order to achieve the dielectrophoretic effect, does not exceed a certain level of about 2 V. At higher voltages, the contact between the cells within the chain, i.e., the contact between their membranes, becomes so close that an irreversible intermingling of the membranes takes place. Cell fusion is observed when the protoplasts are subjected to a field pulse of only 20—50 μs duration which is sufficiently strong to bring about breakdown of the cell membranes. This is achieved by the pulse generator. That the membrane breakdown voltage is indeed exceeded at voltages of 15 V for mesophyll cell protoplasts of *A. sativa* is easily demonstrated by the integrated Laplace equation [14].

$$V = 1.5 \cdot E \cdot r$$

According to this equation the voltage, V, across the membrane is proportional to the radius, r (about 20 μm), and the field strength, E, in the case of a spherical protoplast (shape factor $f = 1.5$). The breakdown voltage is about 1 V as demonstrated for a number of different biological membranes [14—20]. Assuming a homogeneous field, the desired field strength of 330 V/cm is reached with an electrode voltage of 6.6 V at an electrode distance of 200 μm. It is immediately obvious that the breakdown voltage is exceeded by application of the single field pulse, particularly in close proximity to the electrode surface [7,8]. Fusion is immediately initiated when breakdown of the membranes has occurred. The typical sequence of events in the fusion process of mesophyll cell protoplasts of *A. sativa* is shown in Fig. 1. In order to monitor the intermingling of the cytoplasms and the vacuoles, fusion between protoplasts stained with neutral red and unstained protoplasts was observed. As shown in Fig. 1, the fusion of *A. sativa* protoplasts leads to the formation of a new spherical cell. The volume of the fusion aggregate was calculated to be 97% of the initial protoplast volumes. The fusion process is completed within 6 min whereas vacuoles, however, do not seem to have fused. In some cases the vacuoles in the fusion product remained clearly separated for about 10—30 min (not shown) and after that the vacuoles appeared to fuse. It is important to note that this technique can provide high yields of two-celled fusion aggregates when the suspension density of the cells is kept low (Fig. 2). The fusion occurs in a synchronous manner, i.e., all cells exposed to the field pulse undergo fusion.

At present, a number of paradoxical situations exist in the interpretation of the molecular events involved in membrane and cell fusion because of the bewildering diversity of different methods and experimental conditions which ultimately lead to the same result, i.e., fused cells [1—5]. The controversial results in some cases (e.g., as regards the possible role of Ca^{2+} in fusion) indeed make it difficult both to generalise the results obtained to date and to develop a concept for membrane and cell fusion which would be applicable to the

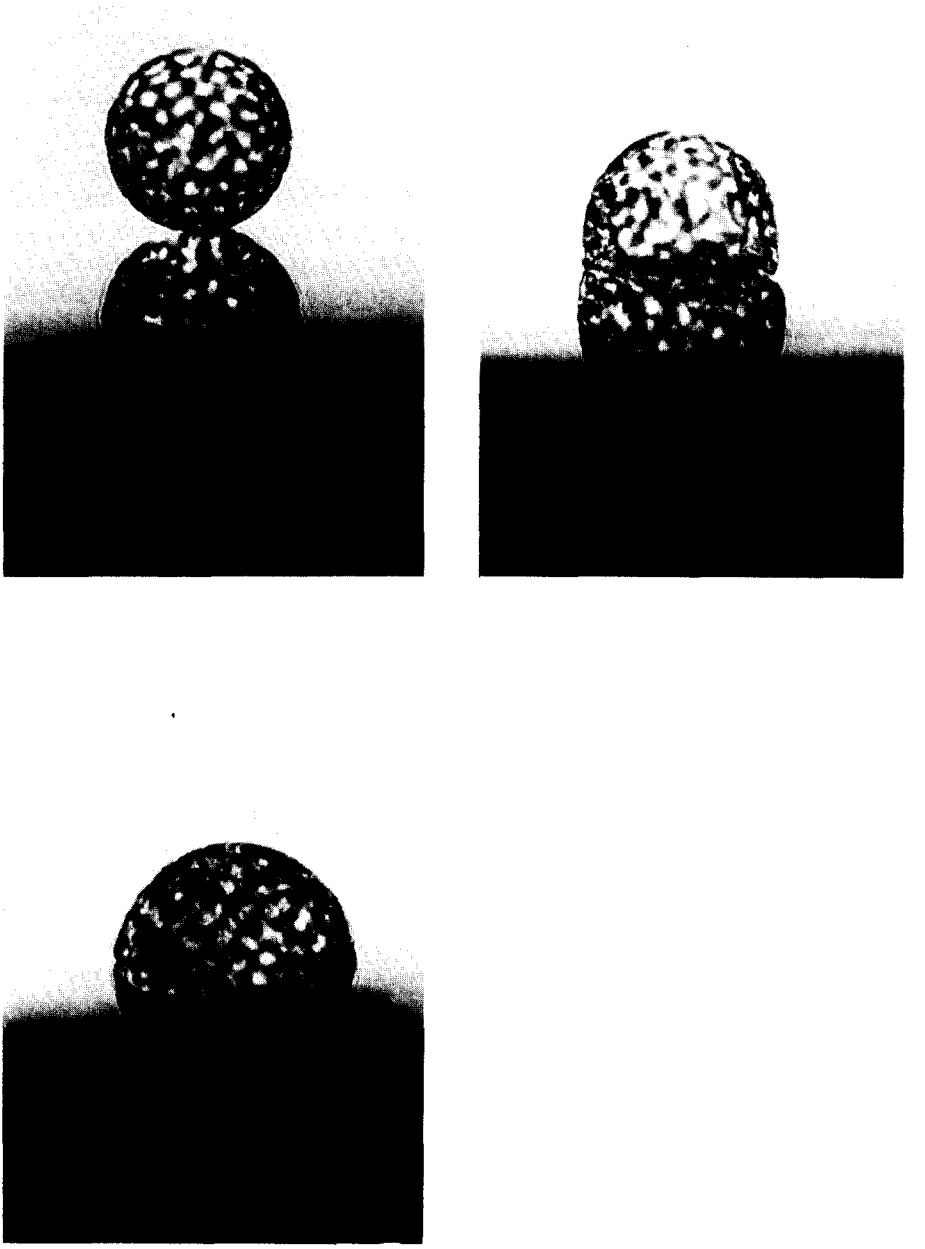

Fig. 2. Formation of a binucleate protoplast of *Avena sativa* by electrically induced cell fusion. Experimental conditions are the same as described in Fig. 1, except that the suspension density was lower. The photographs show two dielectrophoretically adhered protoplasts (top left) and the same aggregate 1 (top right) and 3 min (bottom) after application of the pulse.

fusion process in all living cells.

We believe that the results presented here will shed some light on the ununknown mechanism of fusion. We have demonstrated that an electrical field

pulse of very short duration, but high intensity, can trigger the cell-to-cell fusion process between mesophyll cell protoplasts of *A. sativa*, provided that close membrane contact is first established by an alternating and non-uniform electric field of low intensity.

If we consider these results and bear in mind some recent results of fusion of red blood cells, vacuoles, liposomes and mammalian cells (unpublished results), we can postulate that changes in the intrinsic electric field of the cell membrane is the crucial step in the initiation of cell-to-cell fusion.

It is quite conceivable that reversible electrical breakdown occurs naturally in membranes when the local electrical field, which may be of the order of 10^5—10^8 V/cm, is changed by the addition of a substance (e.g., Ca^{2+}). Because of the rapid resealing time, which Benz and Zimmermann [21] have recently shown to be of the order of 2 μs in lipid bilayer membranes, the original membrane resistance and permeability are immediately restored. As Pethig [8] pointed out, such a process could trigger a number of other membrane processes. However, Pethig was not aware of the bulk of the literature concerning electrical breakdown when he postulated such effects within the biological membrane. This conclusion is along the same lines as the conclusions drawn by several other authors (cf. Ref. 22), but never before has there been such clear-cut evidence for the role of the electric field in the fusion process.

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