

Fusion of bull and human sperm with *Nicotiana* protoplasts

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Bull and human spermatozoa were fused with *Nicotiana tabacum* leaf protoplasts by using polyethylene glycol (PEG). After fusion the cell components were cultured for 20 and 48 hours. Electron microscopy and Nomarski optics were used to control the process. After fusion between bull sperm and protoplasts the following was observed. (1) One or several spermatozoa penetrated into single protoplasts. (2) In electron microscope sections the sperm heads can be seen to be in contact with the plant organelles. (3) Nuclear fusion resulted in one or several sperm nuclei fusing with the nuclei of *Nicotiana* and the chromatin of the sperm decondensing. (4) The fusion also occurs without PEG under spontaneous conditions. The human sperm also penetrates into the protoplasts and can be seen in E.M. sections in contact with the chloroplasts. The fusion also occurs under spontaneous conditions when living human spermatozoa are mixed with protoplasts. There are several similarities between this type of fusion and fertilization occurring in animals. The implications of the experiments are discussed.

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Our first set of experiments on fusion of human and plant cells was carried out between HeLa cells and *Daucus carota* protoplasts (DUDITS et al. 1976). In that study, conditions generally favouring the plant cell were employed: plant culture medium, room temperature, and polyethylene glycol as fusion agent. The two types of nuclei could be recognized in the heterokaryon by differential staining and by tritium labelling of the HeLa cells. Seventy-two hours after fusion the cells appeared surrounded by cell walls and in some cases contained fused interphase nuclei of HeLa and carrot. The percentage fusion was 0.3 to 0.6 %.

A second set of experiments was carried out, the conditions of which instead favoured the human cells: human body temperature (37°C), human cell culture medium and Sendai virus as fusogen (LIMA-DE-FARIA et al. 1977). A plant cell culture known to withstand high temperatures was employed. Cells of *Haplopappus gracilis* grow

well at temperatures above 30°C. Before fusion, the nucleus and cytoplasm of the human cells were labelled with ³H-thymidine and ³H-uridine. Fusion led to the formation of heterokaryons containing one human and one *Haplopappus* nucleus, which appeared with a frequency of 12 %. Heterokaryons with several HeLa and *Haplopappus* nuclei were also present in the preparations. After three days in culture, human chromosomes could be seen dividing in the mixed plant-human cytoplasm. In the heterokaryons a cell wall had formed and had surrounded the two types of cytoplasm that had become mixed (LIMA-DE-FARIA 1981).

The question then arose, what experiment could be made which would permit an efficient development of the fused cell components. Somatic cells that had been used in the two previous experiments could not be expected to develop in culture for a long time. We then contemplated the possibility of using cells which would allow the creation of complete individuals. The sperm represents the germ line on the mammalian side and from a single tobacco cell grown in culture it is possible to develop a complete plant which gives flowers and fruits (VASIL and HILDEBRANDT 1965; SMITH et al. 1976). For this reason we attempted

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the fusion of bull and of human spermatozoa with *Nicotiana tabacum* protoplasts.

Materials and methods

1. Bull and human sperm

Bull (*Bos taurus*) seminal fluid was obtained from the insemination station at Hörby, Sweden. The fluid was transported in a thermos flask at ambient temperature and reached the laboratory within three hours. To obtain clean sperm the seminal fluid was purified following RUDAK et al. (1978) and GLEDHILL et al. (1972). The procedure, carried out under sterile conditions, consisted of adding three volumes of Hanks culture medium lacking potassium and glucose, pH 8.1, (30°C) to one volume of fluid, and then filtering the mixture through a double layer of Kleenex medical wipes.

The filtrate was centrifuged at 455 g for 5 min in a Sorvall GLC-1 centrifuge, using the rotor HL-4.

The pellet was resuspended in the same medium and recentrifuged. To the resedimented pellet was added: 0.4 M glucose, 3.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.7 mM KH_2PO_4 , pH 4.6. The sperm was resuspended and centrifuged once more, and the pellet finally resuspended in the latter solution.

Human seminal fluid obtained from anonymous donors (who were not patients) at the Urology Clinic of the University Hospital was treated similarly.

2. Protoplasts of *Nicotiana tabacum*

Two-month-old greenhouse-grown plants were placed in the dark in a cold room (4°C) the night before the experiment. The protoplasts were isolated by flotation following SHEPARD and TOTTEN (1975) and SHAHIN and SHEPARD (1980). Leaves were surface-sterilized in a 20 % solution of commercial bleach containing 4.5 % NaOCl for 15 min, followed by a 1 min dip in 70 % ethanol, rinsed three times in sterile distilled water and held at 4°C for 30 min. The abaxial epidermis was removed by peeling and the leaf pieces floated on a medium containing: 0.5 % Macerozyme, 2 % cellulase "Onozuka" R-10 (both enzymes from Yakult Honsha, Japan), 0.3 M sucrose, pH 5.6. To improve penetration of the incubation medium, vacuum (700 mm Hg) was applied for 5 min (twice). Cell wall digestion took place under agitation at 30°C for 40 min. The suspension was then filtered through a 60 μm nylon mesh. To 40 ml of

filtrate was added 10 ml of a solution of 0.6 M sucrose, 0.05 M CaCl_2 , pH 6.6. The suspension was centrifuged (129 g, 10 min) in modified 25 ml-Babcock bottles. The protoplasts were transferred to sterile centrifuge tubes, and a solution containing 0.4 M glucose, 3.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.7 mM KH_2PO_4 , pH 4.6 was added (3 times the volume of the protoplast solution). The protoplasts were centrifuged at 129 g for 5 min, after which the pellet was resuspended again in the same medium.

3. Fusion of sperm with protoplasts

Polyethylene glycol (PEG) 1500 was used at concentrations of 50 % and 90 % (0.5 g and 0.9 g PEG, respectively, made to 1 ml with 0.1 M glucose). To this solution were added KH_2PO_4 (0.7 mM) and CaCl_2 (10.5 mM) (KAO and MICHAYLUK 1974; ELSEVIER and RUDDLE 1976). Fusion was carried out in plastic petri dishes. Three drops of protoplasts and a similar volume of sperm were placed side by side and mixed; 18 drops of PEG solution were slowly added dropwise. The treatment with PEG lasted 40 min. After that, a protoplast culture medium was added in a ratio of 1:1 (MURASHIGE and SKOOG 1963, 0.3 M with respect to sorbitol and 0.03 M with respect to sucrose and glucose; pH 5.8). After 10 min the fused cells were collected and centrifuged at 129 g for 5 min. The pellet was resuspended in culture medium and the suspension centrifuged again. Following this procedure the pellet was resuspended once more in culture medium and the cells were pipetted into a plastic petri dish sealed with Parafilm. The plastic petri dish was placed in a large glass petri dish containing moistened filter paper and put under light at 24°C. The fused cells were in culture for 20 and 48 h, after which they were sedimented and fixed.

4. Fixation and staining for light and electron microscopy

For light microscopy the pellet was fixed in acetic acid—alcohol 1:3. Two procedures were employed: carbol-fuchsin for staining the nucleus and cytoplasm, and the Feulgen reaction to localize specifically the nuclei. The cell components were spread on gelatin-coated slides with siliconized Pasteur pipettes, and gently airdried. The carbol-fuchsin stain was that used by BIANCHI et al. (1964), and the staining procedure was similar to that described earlier by LIMA-DE-FARIA et al. (1977). The Feulgen reaction involved washing of

the stained slides in SO_2 -water (three passages) to ensure a specific DNA reaction. All slides were mounted in neutralized Canada balsam.

Following fusion some of the samples were treated with 0.25 % trypsin solution for 10 min to remove spermatozoa that were adsorbed to the cell surface (SAWICKI and KOPROWSKI 1971). The material was centrifuged and the pellet prepared.

The fixation for electron microscopy was as follows: The cell components were resuspended in 0.6 M sorbitol, 0.1 M glucose and 0.05 M CaCl_2 , pH 6.4, and recentrifuged (129 g, 3 min).

The pellet was fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h at 4°C. After sedimentation, (129 g, 5 min) the cells were resuspended in the same buffer and kept for 10 min in the cold. The washing was repeated and the cells were kept overnight in a refrigerator. Following centrifugation the pellet was resuspended in 2 drops of 0.1 M veronal-HCl buffer (pH 7.2) in an Eppendorf centrifuge tube (volume 1 ml). After another centrifugation 5 ml of 1 % OsO_4 in 0.1 M veronal-HCl buffer (pH 7.2) were added and the pellet kept on ice for 30 min. The cells were then washed 3 times in the same buffer. An equal volume of molten 2 % agarose was added to the buffer present over the pellet (2 drops), the contents were agitated by vortex mixing, centrifuged at 1821 g for 5 min, and placed on ice for 30 min. The Eppendorf tube was cut at its base to extract the agarose mounted cells. The block was sliced into smaller pieces with a razor blade and the pieces dehydrated in a graded ethanol series. Embedding was carried out in a mixture of epon and araldite.

Sections were cut at 60–90 nm (silver to gold) with glass knives prepared with the help of the LKB Knife-Maker 7800B. Sectioning was performed with the LKB Ultratome III 8800A. The sections were collected on copper grids (200 mesh) coated with a formvar and a carbon film, and stained with uranyl acetate (5 % uranyl acetate, 90 % ethyl alcohol, 1:1) in darkness for 15 min. This procedure was followed by lead citrate staining at 1:100 concentration for 5 min.

Micrographs were taken using the Jeol Jem-100B (resolution 2–3 Å) electron microscope at magnifications of 3,000 to 7,000 and were then enlarged photographically.

5. Observation by Nomarski differential interference contrast

Protoplasts of *Nicotiana tabacum* were mixed with moving cleaned sperm in 0.4 M glucose, 3.5

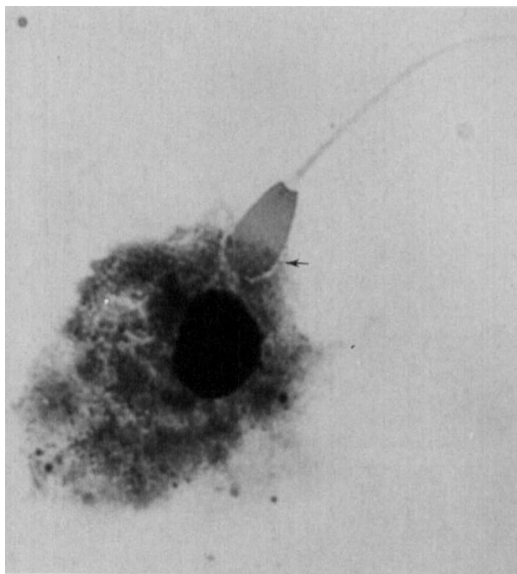


Fig. 1. Bull sperm penetrating a *Nicotiana* protoplast following PEG treatment. There is a band of light material surrounding the frontal part of the sperm head (arrow). The fixed material has been air dried on a slide and stained with carbol-fuchsin. The protoplast has a deeply stained nucleus. $\times 1,624$.

mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.7 mM KH_2PO_4 , pH 4.6, and observed and photographed directly with Nomarski differential interference contrast optics (A-Nic Vanox, Olympus). This mode of microscopy produces high-contrast and relief-like images of the unstained, transparent protoplasts and sperm cells in a wide range of interference colours.

Results

The use of Babcock bottles in combination with the solutions employed permits obtention of an upper layer of pure protoplasts. All the leaf debris are collected at the lower part of the tubes. Most of the protoplasts of *Nicotiana tabacum* obtained by this procedure contain chloroplasts situated at the periphery and have the central region filled by a large vacuole. The cell nucleus is also usually located near the cell membrane (Fig. 2–3).

At the same time the cleaning of the seminal fluid results in a pellet of pure and active spermatozoa (Fig. 4).

The presence of these two clean cell types is a prerequisite for a perfect fusion between them.

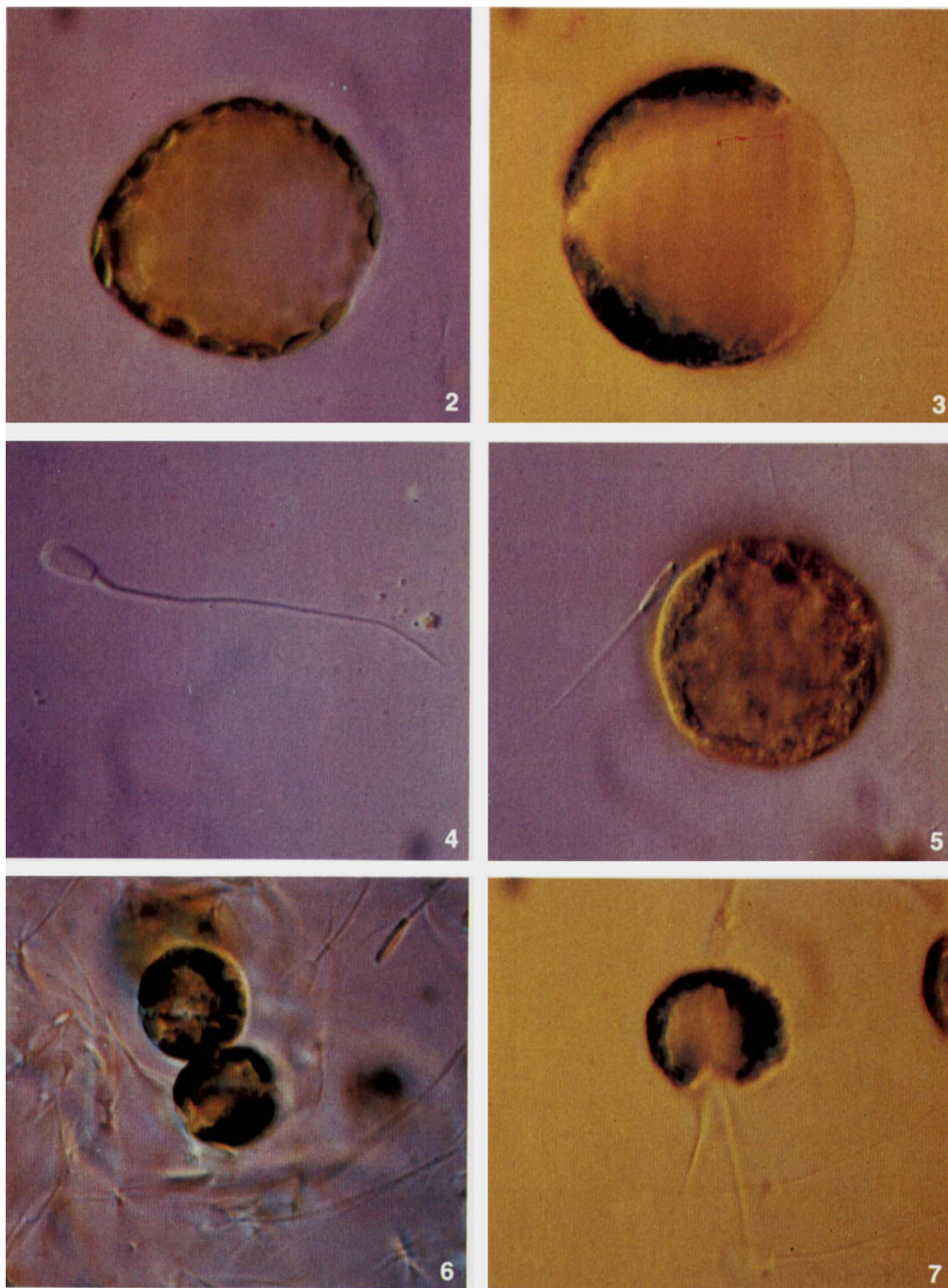


Fig. 2–7. *Nicotiana tabacum* protoplasts and bull sperm photographed in vivo under Nomarski illumination. **Fig. 2 and 3.** Single protoplasts showing the peripheral location of the chloroplasts and the large vacuole situated in the central region. **Fig. 4.** A single bull sperm with its characteristic large and flat head seen from above. **Fig. 5.** A bull sperm (with the head seen from the side) in contact with a protoplast. **Fig. 6.** Following PEG treatment two united protoplasts are surrounded by many spermatozoa forcing their way into a protoplast by pushing the cell membrane with their heads. **Fig. 7.** Two spermatozoa forcing their way into a protoplast by pushing the cell membrane with their heads.

Moreover, the pellet of clean spermatozoa was diluted in the same medium in which the protoplasts are maintained. This permitted the mixture of the two cell types without any harm to the protoplasts and it turned out that the spermatozoa became extremely active in this solution (Fig. 5-7).

Induced fusion of bull spermatozoa with *Nicotiana* protoplasts

Fusion was induced with PEG (Fig. 6). With carbol-fuchsin staining, plant nuclei are easily distinguished from the sperm nuclei. The former stain an intense red-violet whereas the latter are coloured homogeneously light blue.

The protoplasts are well preserved and surrounded by numerous spermatozoa. There is fusion between protoplasts as well as between protoplasts and sperm. Fusion between two and up to six protoplasts was evident in many instances. In these cases spermatozoa were trapped between the protoplasts, a factor that may have aided their penetration into the plant components. However, there are many instances of spermatozoa also penetrating solitary protoplasts.

Spermatozoa inside the plant cell are distinguishable from those outside by the difference in head morphology as well as by other features. The sperm heads outside the protoplast retain very sharp outlines whereas those inside the protoplast become diffuse since they are masked by the cytoplasm and the cellular membranes. The same is true for the tails. Some of the heads become deformed following penetration, as a result of fusion with cell components such as the nuclei (Fig. 12 and 13). The penetration of a sperm into a protoplast is accompanied by the formation of an aureola around the frontal part of the sperm head, i.e. a light band is seen surrounding this part of the head of the spermatozoon when it enters the plant cell (Fig. 1).

It is difficult to exactly estimate the frequency of fusion, for the following reasons: (1) penetration of both sperm heads and tails into the protoplasts, (2) difficulty of recognizing tails as a result of their dimension and form, and (3) obscuring of spermatozoa by chloroplasts. However, the estimated percentage of fusion was high, of the order of 20 % and often includes several spermatozoa per plant cell (up to five).

This was confirmed by electron microscopy, where most sections viewed showed protoplasts into which one or several spermatozoa had pene-

trated. This is taken as an indication that the figure mentioned above is probably an underestimation.

Feulgen staining permitted to analyse the fate of the sperm. Once inside the protoplast, the sperm tails separated from the heads and these fused with the plant nuclei. The sperm nucleus partly disappears as it combines with the plant nucleus (Fig. 17-21). In a few cases two sperm nuclei were observed to penetrate a plant nucleus. The chromatin of the sperm nucleus becomes partly decondensed during the fusion process (Fig. 19 and 20).

In electron microscopy the sperm heads are recognized by their characteristically dense staining, and the tails, by their symmetrically patterned central filaments. In Fig. 22-24 one to several sperm heads and tails are seen inside the protoplasts in intimate contact with the nucleus and chloroplasts of the plant cell.

Spontaneous penetration of bull sperm into *Nicotiana* protoplasts

It is possible to observe the penetration of bull sperm into protoplasts of *Nicotiana* also in the absence of PEG. A mixture of the two cell types observed by Nomarski interference contrast shows the penetration of one or several spermatozoa into single protoplasts maintained in the medium.

It can be easily demonstrated that the spermatozoa are inside the living plant cell because they move rapidly within the vacuole and cytoplasmic components against the chloroplasts without being able to escape from the protoplast. As the sperms wave their tails they get out of focus of the microscope. Fig. 14-16 represent different focal planes of spontaneous fusion within about 20 sec. intervals.

Two protoplasts lie adjacent to each other; the one on the left was penetrated by two sperm, the other also by two which, however, appear in focus at different instances due to their continuous movement.

The percentage (4 %) of spontaneous fusion was higher than expected. It might be supposed that spontaneous penetration was enhanced by disruption of the plasmalemma in some protoplasts. However, this appeared not to be the case. Sperm could be observed beating against the plasmalemma (as many as 10 times) until they finally penetrated the protoplast. With the head inside the protoplast, the tail (which is longer than the diameter of most protoplasts) may remain partly

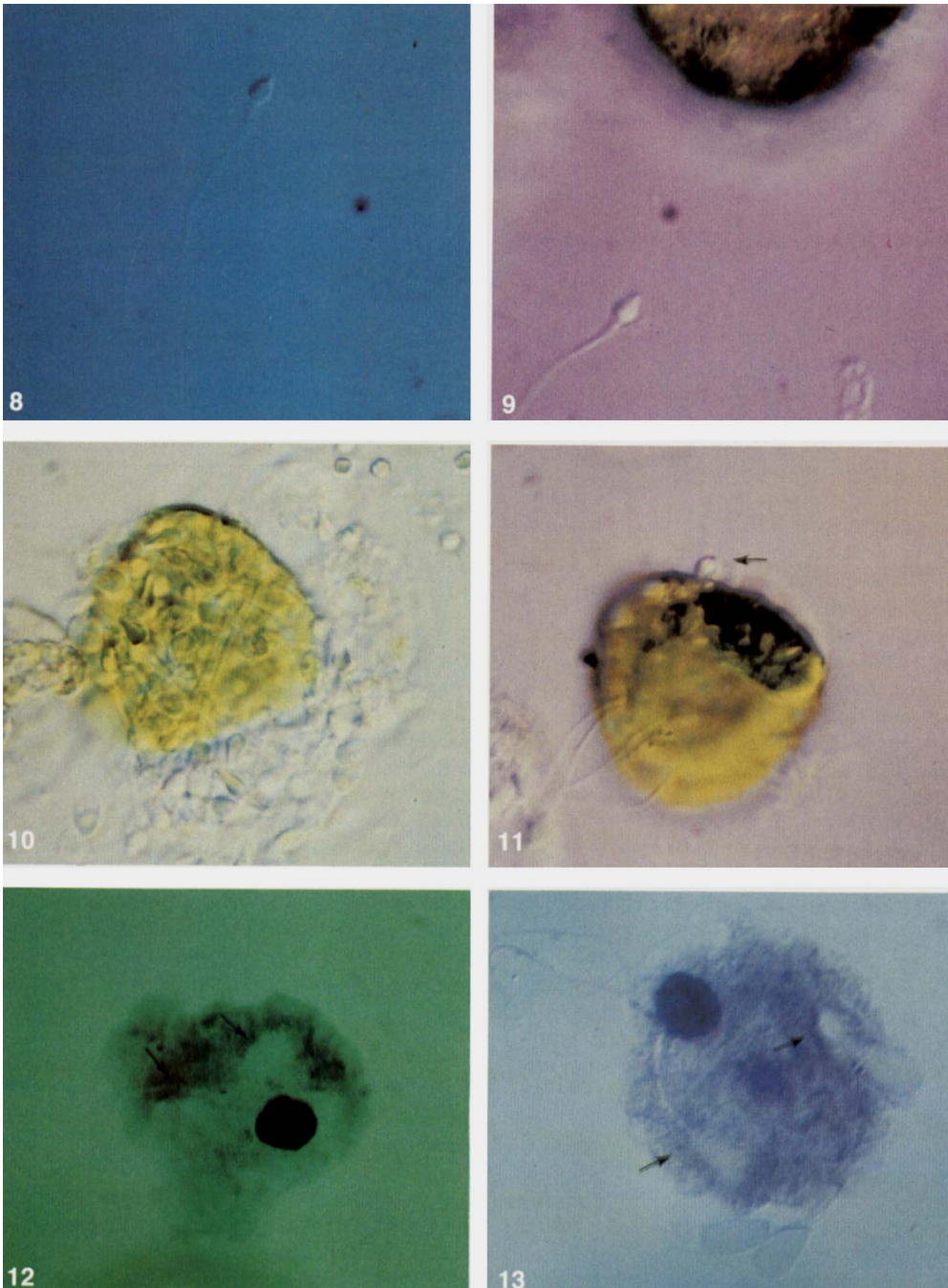


Fig. 8–13. Fig. 8–11. Human sperm and *Nicotiana* protoplasts photographed in vivo with Nomarski illumination. Fig. 8. A single human sperm with its small conic head (seen from above). Fig. 9. A human spermatozoon approaching a plant protoplast. Fig. 10. *Nicotiana* protoplast (with its many chloroplasts) surrounded by numerous spermatozoa. Fig. 11. The head of a human sperm trying to penetrate a protoplast (arrow). Fig. 12 and 13. Bull sperms which have penetrated into *Nicotiana* protoplasts following PEG treatment. Cells air dried and stained with carbol-fuchsin (the plant nucleus is deeply stained). Fig. 12. The tail and the head of a spermatozoon can be seen inside the protoplast as a lighter stained component (arrows). Fig. 13. Several sperms are outside, others have partly or totally penetrated into the protoplast (arrows).



Fig. 14–16. *Nicotiana* protoplasts photographed in vivo with Nomarski illumination. Bull sperms have penetrated into them spontaneously and keep moving within the central vacuole of the protoplast. **Fig. 14.** Two sperms appear inside the protoplast situated on the left, one of them is in full focus. **Fig. 15.** The sperms of the left protoplast get out of focus due to their movements, and one sperm on the right protoplast appears now in focus. **Fig. 16.** Two spermatozoa which have penetrated the protoplast on the right have moved suddenly into focus. It can be checked that the sperms are inside the plant cell because they "swim" inside it without being able to get out.

outside, or it may enter the plant cell in its entirety.

Induced fusion of human sperm with *Nicotiana* protoplasts

Human sperm compared with bull sperm is characterized by a smaller head and tail. The head is also slightly conic (Fig. 8–11). The result of the fusion was essentially similar to that observed with bull sperm. Light and electron microscopy revealed the presence of human sperm inside the protoplasts and in contact with the chloroplasts (Fig. 25 and 26).

Spontaneous penetration of human sperm into *Nicotiana* protoplasts

A mixture of plant protoplasts and human sperm was observed by Nomarski interference contrast. The percentage of spontaneous fusion (about 0.1 %) was lower than in the case of bull sperm, but still appreciable if one takes into consideration the smaller amount of spermatozoa present in the mixture. Once inside the protoplast the human sperm moves vigorously with its head striking against the cell contents.

Discussion

Comparison of animal fertilization with the fusion between sperm and protoplasts

Are there any similarities between interkingdom fusion, as observed in these experiments, and fertilization in animals? Some apparent similarities are the following:

- (1) During penetration of the bull spermatozoon into the protoplast of *Nicotiana* the sperm forms an aureola around the frontal part of its head. This is similar if not identical to the penetration of sperm into the egg of *Hydroides* (COLWIN and COLWIN 1967).
- (2) After penetration into the plant cell the tail of the sperm is detached from its head. This is a phenomenon described during the fertilization of *Arbacia* eggs (AUSTIN 1965).
- (3) In some cases several spermatozoa penetrate the protoplast. This is a phenomenon similar to polyspermy, a normal process in certain species such as *Triton* (Urodeles) (AUSTIN 1965).

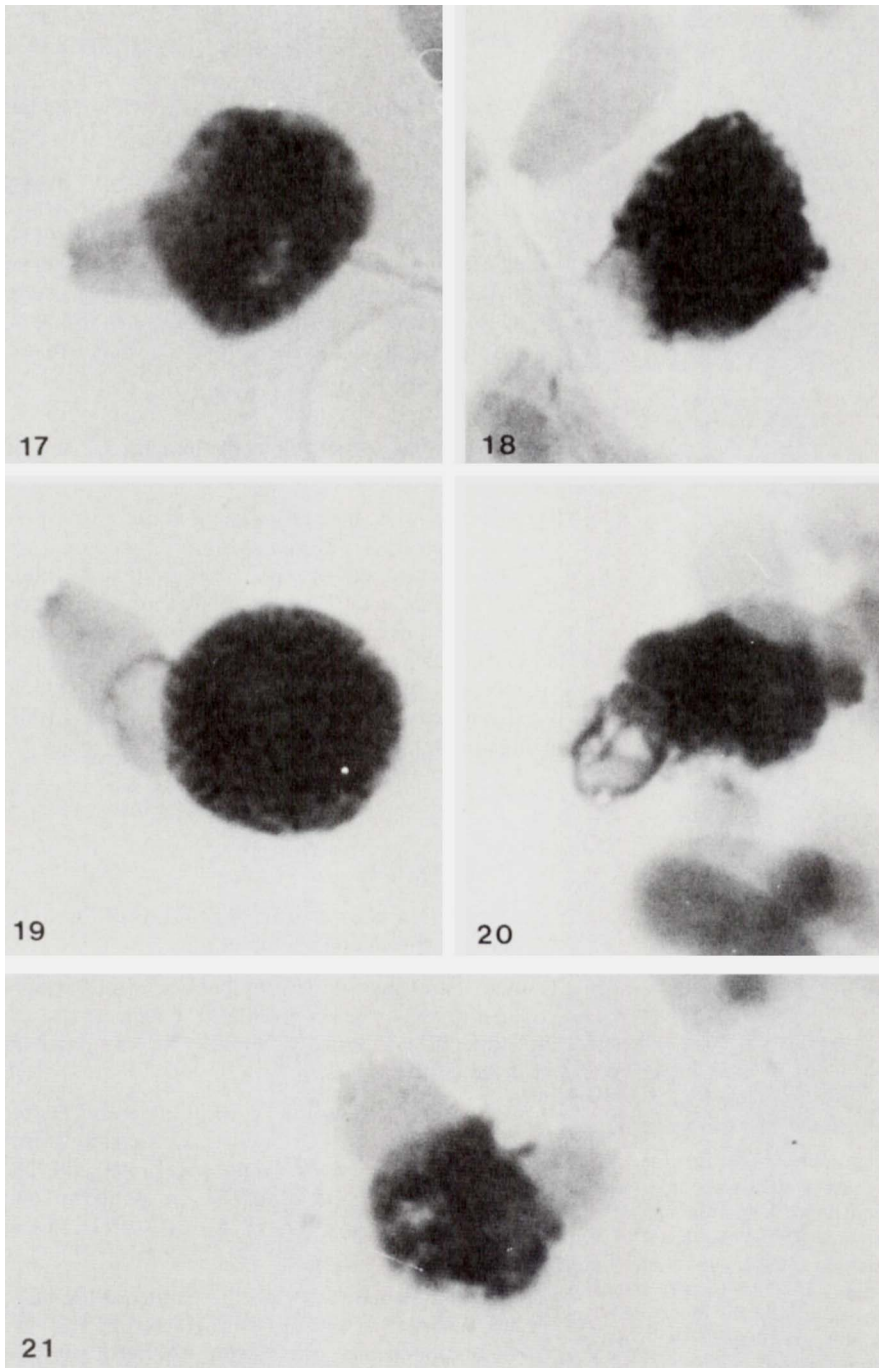


Fig. 17–21. Nuclear fusion. Bull sperm fused with *Nicotiana* protoplasts following PEG treatment. The cellular components were stained by the Feulgen reaction to allow to see the nuclei distinctly. The plant nucleus is deeply stained, the sperm head is slightly stained. The tails of the sperms have detached from the heads. **Fig. 17 and 18.** The animal and plant nuclei have fused, one of the sperm nuclei has nearly disappeared into the plant nucleus (**Fig. 18**). **Fig. 19 and 20.** As the sperm and plant nuclei fuse the chromatin of the sperm starts to decondense. **Fig. 21.** Two sperm heads have fused with one plant nucleus. $\times 3,500$.

(4) There is not only cell fusion but also nuclear fusion. The nucleus of the bull sperm can be seen to fuse with that of the plant cell. Fusion of sperm and egg nuclei is a common feature of animal fertilization, but it does not occur in every species. In certain organisms the nuclei remain side by side without fusing (AUSTIN 1965). Thus, the fact that the plant and animal nuclei fuse is more similar to the general type of fertilization than to certain types of animal fertilization.

(5) The chromatin of the nucleus of the bull sperm changes in structure after entering the plant cell, becoming dispersed and vacuolized. This is also the case in human fertilization. After the human sperm enters the egg its chromatin decondenses (BARROS and FRANKLIN 1968; KVIST 1980).

(6) Fusion can be induced by PEG, but also takes place spontaneously. This makes the process still more similar to a normal fertilization.

Sperm-protoplast fusion and fertilization in lower plants

The male gametes of flowering plants are incorporated in the pollen grains. These withstand the dry conditions of transportation by air, insects and birds. Spermatozoa need water or a humid environment, such as the mammalian vagina to survive. In aquatic plants such as algae, fertilization involves sperm cells that are very similar to mammalian sperm.

(1) The tails of the spermatozoa of algae and of mammals, including humans, have similar structural features, as revealed by electron microscopy. The tail is constructed of filaments organized in the same basic pattern (AFZELIUS 1969).

(2) The egg cell of an alga (the oosphere) such as that of *Fucus* has no wall, but only a membrane. The cell wall only forms after fertilization (STRASBURGER 1924). The bull and human sperm when entering a protoplast confront a cell that structurally is similar to that which the algal sperm faces when it fertilizes the oosphere.

For this reason the fusion between human sperm and *Nicotiana* protoplasts is not functionally novel but recreates a situation common to plants before their evolution into land forms.

Spontaneous penetration

The spontaneous penetration observed between bull and human sperm and plant protoplasts is not

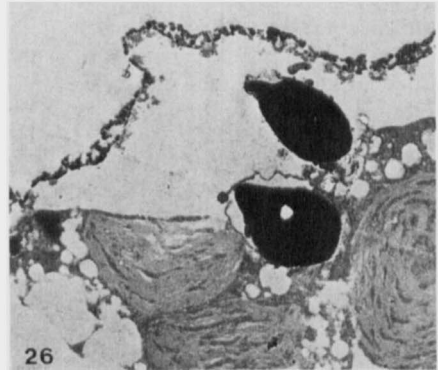
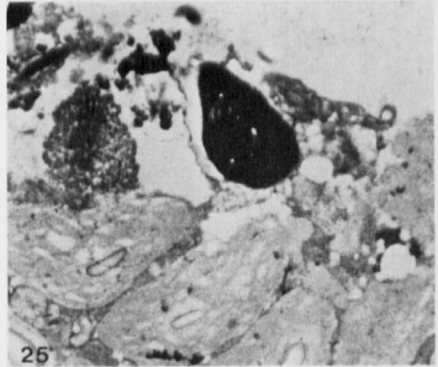
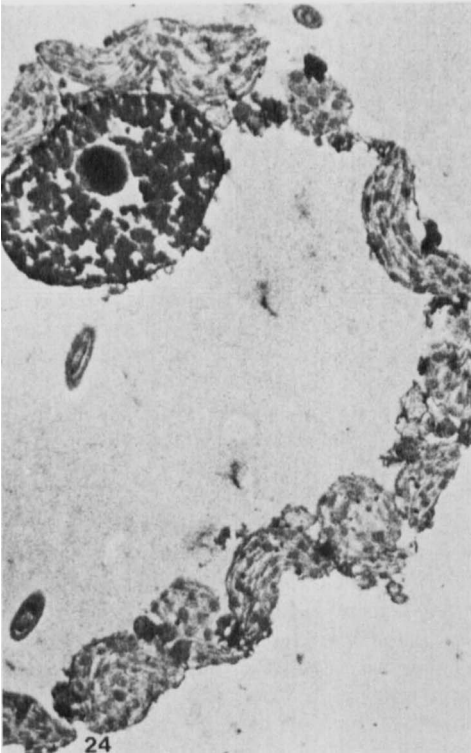
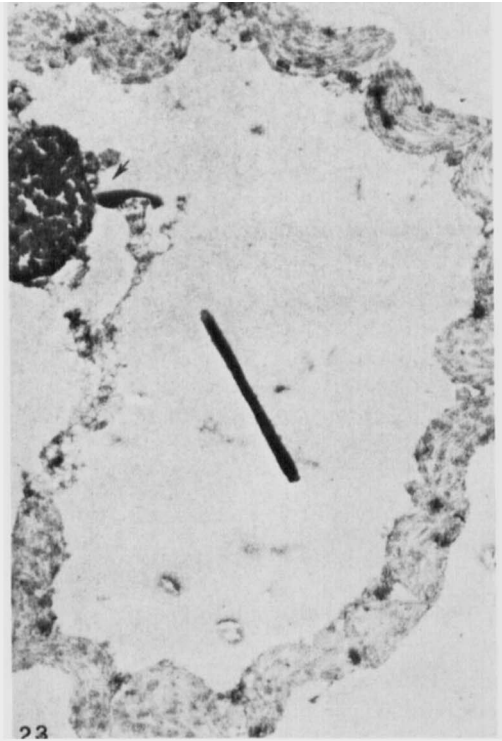
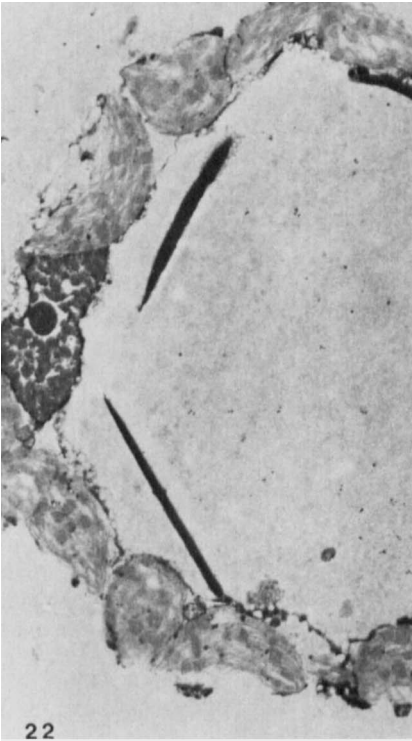
exceptional. Spontaneous fusion has been observed between sperm cells and mammalian somatic cells (BENDICH et al. 1974; PHILLIPS and PHILLIPS 1974). An animal somatic cell is structurally similar to a protoplast in that it has no cell wall.

Implications of the experiments

In our previous experiments of the fusion between human and plant cells (DUDITS et al. 1976; LIMA-DE-FARIA et al. 1977) the fused cell components have been kept in culture for 3 days. The question that arose was for how long time could these cells be maintained, and how long would they survive? Instead of culturing further these cells we looked for a more radical solution. The use of the germ line on the human and plant side would give a better chance to improve those conditions. As mammalian germ line we chose the sperm, and as plant germ line we chose the leaf protoplasts of *Nicotiana tabacum* because a complete plant, which gives flowers and fruits, can be obtained from a single tobacco cell kept in culture (VASIL and HILDEBRANDT 1965). The introduction of a nucleus from a bull or a human sperm into a plant cell opens the way for the production of complete plants containing genetic components of human or mammalian origin.

There are four methods that have been considered as possible systems of transferring genetic information from one cell to another: (1) somatic cell hybridization, (2) viral transformation, (3) incorporation of material from isolated chromosomes and (4) entry of sperm into animal somatic cells (PHILLIPS and PHILLIPS 1974). The potential value of this last system has been emphasized by these authors. DNA synthesis was initiated in rabbit spermatozoa after these fused with hamster somatic cells (GLEDHILL et al. 1972). In our experiments the chromatin of the bull sperm decondenses when it fuses with the plant nucleus, and there are other features similar to a normal fertilization.

As pointed out earlier (LIMA-DE-FARIA 1977) there is nothing especially dramatic in this experiment. Humans contain genes which are common to plants, and plants contain several genes that before were considered typical of humans or mammals. If one introduces an hemoglobin gene into a plant, this should not represent a genetic innovation since plants of the family Leguminosae contain the genes which produce leg-hemoglobin. This hemoglobin has an amino



acid sequence similar to that of humans (ELLFOLK and SIEVERS 1971; ELLFOLK 1972). The same would be true for the introduction of an insulin gene. Insulin is produced by the cells of *Momordica charantia*, which belongs to the Cucurbitaceae family (KHANNA et al. 1974). The introduction of a gene for cytochrome C would also not be completely foreign to a plant genome since the cytochrome C of plants is not very different from that of humans (DAYHOFF 1971).

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Fig. 22–26. **Fig. 22.** Electron micrograph of a section of a protoplast which contains the densely stained heads of three bull spermatozoa. Note the chloroplasts situated at the periphery of the protoplast and the plant nucleus with its nucleolus (at 9 o'clock). $\times 4,752$. **Fig. 23.** Two bull sperm heads (deeply stained) inside a protoplast. One of them is in contact with the plant nucleus (arrow). Electron micrograph $\times 5,350$. **Fig. 24.** Two bull sperm tails inside and one outside a protoplast which has a nucleus with a distinct nucleolus (11 o'clock). Electron micrograph $\times 5,140$. **Fig. 25 and 26.** Human sperm heads inside *Nicotiana* protoplasts and at the side of the chloroplasts. The vacuolated aspect of the deeply stained heads is characteristic of spermatozoa. $\times 7,232$.