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Fusion characteristics of plant protoplasts in electric fields

M.J. Tempelaar* and M.G.K. Jones
Department of Biochemistry, Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ, UK

Abstract. The electrical parameters important in the fusion of plant protoplasts aligned dielectrophoretically in high-frequency alternating electric fields have been established. Protoplasts were aligned in an alternating electric field between two relatively distant (1 mm) electrodes, by dielectrophoresis induced by field inhomogeneities caused by the protoplasts themselves. This arrangement allowed ease of manipulations, large throughput and low loss of protoplasts. In analytical experiments, sufficiently large samples could be used to study pulse duration-fusion response relations at different pulse voltages for protoplasts of different species, tissues and size (mesophyll protoplasts of *Solanum brevidens, Triticum aestivum, Hordeum vulgare;* suspension-culture protoplasts of *Nicotiana sylvestris, N. rustica, Datura innoxia* and *S. brevidens;* root-tip protoplasts of *Vicia faba,* hypocotyl protoplasts of *Brassica napus*). The percentage of aligned protoplasts that fused increased with increasing pulse parameters (pulse duration; voltage) above a threshold that was dependant on pulse voltage. The maximum fusion values obtained depended on a number of factors including protoplast origin, size and chain length. Leaf mesophyll protoplasts fused much more readily than suspension-culture protoplasts. For both types, there was a correlation of size with fusion yield: large protoplasts tended to fuse more readily than small protoplasts. In short chains (≦five protoplasts), fusion frequency was lower, but the proportion of one-to-one products was greater than in long chains (≧ten protoplasts). In formation by electrofusion of heterokaryons between mesophyll and suspension-culture protoplasts, the fusion-frequency response curves reflected those of homofusion of mesophyll protoplasts rather than suspension-culture protoplasts. There was no apparent limitation to the fusion of the smallest mesophyll protoplast with the largest suspension-culture protoplasts. Based on these observations, it is possible to direct fusion towards a higher frequency of one-to-one (mesophyll/suspension) products by incorporating low densities of mesophyll protoplasts in high densities of suspension-culture protoplasts and by using a short fusion pulse. The viability of fusion products, assessed by staining with fluorescein diacetate, was not impaired by standard fusion conditions. On a preparative scale, heterokaryons (*S. brevidens* mesophyll – *N. sylvestris* or *D. innoxia* suspension-culture) were produced by electrofusion and cultured in liquid or embedded in agar, and were capable of wall formation, division and growth. It is concluded that the electrode arrangement described is more suitable for carrying out directed fusions of plant protoplasts than that employing closer electrodes.

Key words: Cell division – Dielectrophoresis – Electrofusion (electrical parameters) – Heterokaryon – Protoplast (mesophyll and suspension) – Somatic hybridization.

Introduction

Protoplast fusion holds considerable potential for the introduction or combination of useful genetic characters in crop plants at present unattainable by conventional plant breeding (Jones et al. 1984).

The most widely applied methods of protoplast fusion, utilising polyethylene glycol and Ca^{2+} at high pH (Kao and Michayluk 1974; Evans 1984), have drawbacks in that the fusion frequency is

* *Permanent address:* Department of Genetics, Biological Centre, University of Groningen, P.O. Box 14, 9751 AA Haren, The Netherlands

variable and often low, and control of the fusion is limited. More recently, techniques of electrofusion have been developed (Zimmermann 1982), and it has been suggested that they are superior to the chemical fusion methods. In all cases, the electrofusion is induced by a short (<100 μs) electric pulse (10^3–>3.10^3 V cm^{-1}), but there are a number of different strategies in the arrangement and control of the process.

In the method of Zimmermann (Zimmermann and Scheurich 1981; Scheurich et al. 1981; Zimmermann 1982), protoplasts are drawn together in 'pearl chains' and towards the electrodes by dielectrophoresis using an inhomogeneous high-frequency alternating electric field between close parallel wire electrodes (usual separation 150–250 μm). This principle has also been applied in flow systems for plant protoplasts (Bates et al. 1983). However, there may be problems with adherence of plant protoplasts to the wires, damage may occur in long chains, and the electrode chamber capacity is small, thus limiting the number of protoplasts handled in a fusion cycle. The advantage of this electrode arrangement is that, at low cell densities, in theory a high yield of directed one-to-one fusion products should be obtained by sequential introduction of different protoplast fusion partners.

To overcome the difficulties outlined, movement towards the electrodes can be avoided by using weaker alternating fields and–or more widely spaced electrodes. In such situations, cells or protoplasts at high densities are attracted only towards one another by mutual dielectrophoresis caused by the local inhomogeneities they induce in the field: electrofusion can take place anywhere between the electrodes (Richter et al. 1981; Zimmermann 1982; Pilwat et al. 1981; Watts and King 1984). This approach allows the use of a variety of electrode arrangements, such as wires stretched across a Petri dish, or thin metal sheets that can be transferred from one vessel containing protoplasts to another (Watts and King 1984).

A third approach has been applied to animal cells which grow in confluent layers and consequently are in close contact. A direct-current pulse is sufficient to induce fusion, and proven hybrids have been produced in comparatively high frequencies (Teissie et al. 1983; Finaz et al. 1984).

In a further variation involving micromanipulation, individual protoplasts have been fused (Senda et al. 1979; Koop et al. 1983). This approach clearly allows considerable control of the fusion partners, but the numbers to be fused are limited and some practice is required.

Despite the apparently clear physics, some of the responses of plant protoplasts in electric fields cannot be predicted readily, and relevant properties must be established or verified by direct microscopic monitoring of fusion events. These include: i) the effect of protoplast origin and size, ii) the relationship between fusion pulse duration and voltage, and the fusion response, iii) the extent to which one-to-one fusions can be induced in pearl chains, and iv) the tendency towards preferential fusions in mixtures of two protoplast populations.

We have determined these properties for protoplasts from different sources using electric fields with an electrode separation of 1 mm for collecting together and fusion of protoplasts. For controlled fusion of plant protoplasts, in our view, the wider electrode spacing provides clear advantages in terms of protoplast numbers, absence of loss of protoplasts to electrode surfaces, and ease of manipulations. In addition, as demonstrated in the following experiments, the properties of different protoplasts can be utilized to overcome the major drawback of this arrangement, that is, the apparent lack of direct control of specificity of the fusion process in a mixture of protoplasts.

Materials and methods

Isolation and purification of protoplasts

Protoplasts were isolated from *Solanum brevidens* Phil. (No. 2451; Commonwealth Potato Collection, Edinburgh, UK), a diploid wild *Solanum* species (Nelson et al. 1983); barley (*Hordeum vulgare* L. cv. Sundance, supplied by J. Franklin, Rothamsted); wheat (*Triticum aestivum* L. cv. Maris Butler; Plant Breeding Institute, Cambridge, UK); *Nicotiana rustica* L. (supplied by M. Davey, University of Nottingham, UK); *N. sylvestris* Speg. and Comes (mutant R1, resistant to aminoethyl cysteine, Negrutiu et al. 1984 supplied by I. Negrutiu, Brussels, Belgium); *Datura innoxia* Mill. (mutant I–Vl, requiring isoleucine and valine, Horsch and King 1983, supplied by J. King, University of Saskatoon, Canada; *Vicia faba* L. cv. Threefold white (Bolhuis b.v., Groningen); and oilseed rape (*Brassica napus* L. cv. Brutor, supplied by J. Boeman, Nickerson Rothwell Plant Breeders). Shoot cultures of *S. brevidens* were maintained as described by Nelson et al. (1983); cereal leaf protoplasts were obtained from expanded leaves of three-week-old seedlings; wheat suspension cultures originated fom excised immature embryos. Suspension cultures other than wheat were maintained on the medium of Uchimiya and Murashige (1974), and the wheat cultures on the medium of Murashige and Skoog (1962) with 2.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid. Root-tip and hypocotyl protoplasts were isolated 2–4 d after germination of seeds (Tempelaar et al. 1982; Glimelius and Ottosson 1983). The enzymes and sources used for protoplast isolation are summarized in Table 1. Cellulase R10 and macerozyme R10 were purchased from Yakult Pharmaceutical Industry Co., Nishinomiya, Japan; Pectolyase Y23 from Seishu Pharmaceutical Co., Tokyo, Japan; Meicelase from Meiji Seika Kaisha, Tokyo, Japan; and Rhozyme HP150 from Pollack and Poole, Reading, UK. The isolation media

Table 1. Sources of plant material and enzymes used for isolation of protoplasts

	Source			
	Mesophyll	Suspension culture	Root tips	Hypocotyl
Plant material	*S. brevidens* (shoot culture) *H. vulgare* *T. aestivum* (leaves-greenhouse)	*S. brevidens* *T. aestivum* *N. rustica* *N. sylvestris* *D. innoxia*	*V. faba*	*B. napus*
Enzymes				
Meicelase	1.5%	–	–	–
Cellulase R10	–	2%	2%	2%
Rhozyme HP150	–	–	2%	2%
Macerozyme R10	–	0.3%	0.3%	0.3%
Pectolyase Y23	0.1%	0.2%	0.1%	0.1%

in every case contained mannitol 9%, KNO_3 190 mg l^{-1}, $CaCl_2 \cdot 2H_2O$ 44 mg l^{-1}, $MgSO_4 \cdot 7H_2O$ 37 mg l^{-1} and KH_2PO_4 17 mg l^{-1}, pH 5.7, in addition to the enzymes. After incubation in enzyme solution for 3–4 h at room temperature, protoplasts were isolated by passage through sieves (400 µm then 100 µm, 50 µm or 38 µm aperture). The protoplasts were either layered directly onto 18% sucrose or, when cultured, concentrated by an initial centrifugation (5 min, 100 *g*), washed in 9% mannitol, then layered onto sucrose and centrifuged (30 min, 700 *g*). The protoplast band at the mannitol-sucrose interphase was collected (volume about 1 ml), resuspended in 10 ml 9% mannitol, pelleted by centrifugation (5 min 100 *g*) and stored in a small volume of 9% mannitol pH 5.6. In many instances, suspension-culture, root-tip and hypocotyl protoplasts were obtained in sufficient purity simply by sieving, but were usually purified by layering over sucrose, sometimes using a 27% sucrose cushion rather than 18%, and centrifuged as above.

Equipment and conditions for electrofusion

Narrowly spaced electrode system. For initial experiments simple equipment, based on that described by Zimmermann and Scheurich (1981); Scheurich et al. (1981) was constructed, linking a 300 kHz signal generator to electronic circuitry for turning off the alternating field from the generator and for switching on a direct-current voltage from a regulated power supply for a continuously adjustable period of 10–300 µs. With this system connected to electrodes on a microscope slide, a number of experiments were carried out with potato leaf protoplasts (monohaploid 7322) in an inhomogeneous-field system as described by Zimmermann (1982). The protoplasts were introduced between two parallel nickel-chromium-coated wires (0.1 mm diameter), 100–200 µm apart.

A collecting field of 30–80 V cm^{-1} peak-to-peak was used and the fusing pulse had a duration of 15–60 µs and an amplitude of up to 3500 V cm^{-1}. Fusion was monitored by analysis of videorecordings of the microscope image of the electrode chamber after different times.

Widely spaced electrode system. This series of experiments was performed with more versatile electronic equipment and modified electrode arrangements. A fusion generator was built by the Central Electronic Department of the University of Groningen, incorporating an oscillator with a range of up to 1 MHz and 1–20 V peak-peak sinewave amplitude, and a pulse generator with calibrated stepwise control of pulse width (10 µs-1 ms) and amplitude (40–200 V). Pulses could be repeated and the field switched off during and after the pulse for variable periods. Pulse durations of >50 µs were effected by a series of shorter pulses delivered at 1-ms intervals. (In tests on pulse duration, the same fusion yields were obtained for single and multiple pulses of equal total length).

For analytical experiments, a sample of protoplasts was introduced between two parallel wires of 0.1 mm diameter, 20 mm length and 1 mm separation. Chains of protoplasts were lined up by a 1-MHz, 8-V, sine wave (80 V cm^{-1}).

After application of the pulse(s) (1000–2000 V cm^{-1}, 10–200 µs) the alternating-current field was reduced routinely to 40 V cm^{-1} to avoid characteristic post-pulse displacements of the protoplasts, but this field strength was sufficient to keep the chains aligned.

Normally, 200–400 protoplasts were introduced into the chamber and more than 50% of these became aligned in the field. Experiments were monitored under the microscope (400-fold magnification) and either scored directly or from videorecordings. The alignment of protoplasts and the formation and composition of fusion products were scored within 15 min of application of the fusing pulse at room temperature.

For fusion of larger numbers of protoplasts, several electrode configurations were tested, including transferable wire electrodes, batch and flow systems. In all cases the 200-V output of the pulse generator limited the electrode separation to a maximum distance of about 1 mm. There were also constraints on surface areas of the electrodes: tests indicated that excessive surface areas imposed too large a capacitive load on the generator output, causing the voltage to drop. We used parallel-sided brass strips 1 mm apart for a batch-fusion chamber and a flow chamber; this electrode composition did not appear to affect culture adversely. Both of these arrangements allowed alignment and fusion to be monitored microscopically. Using these electrodes, protoplasts at a density of $4 \cdot 10^5$ protoplasts ml^{-1} could be aligned quickly into chains of sufficient length to give good yields of fusion products. The whole procedure took 30–60 s.

Viability and cell-wall formation

The viability of protoplasts and electrofusion products was examined by staining with fluorescein diacetate (Larkin 1976) and monitoring for fluorescein accumulation by UV fluorescence (Olympus BH2 microscope; Olympus Optical Co., Tokyo, Japan, fitted for epifluorescence, excitation filter Olympus B (IF-490), dichroic mirror 'B' (DM500+0515) and barrier filter 0530). Cell-wall formation was similarly monitored with 0.01% calcofluor M2R (kindly supplied by Dr. L. Fowke, University of Saskatoon, Canada) (Olympus excitation filter U (UG1), dichroic mirror U (DM400+L420), barrier filter Y435).

Sterile culture of electrofused protoplasts

For sterile culture of protoplasts, solutions of mannitol and sucrose, and all equipment, were sterilized by autoclaving (121° C, 103 KPa, 20 min). The electrode chamber was sterilized by immersion in 70% ethanol, and all manipulations were carried out in a laminar-flow cabinet. Following electrofusion in mannitol, protoplasts were pipetted into 3.5-cm-diameter Pe-

tri dishes to which filter-sterilized culture medium was added in the ratios: 1 to 5, 10, 15, 20, 25 and 30. The culture media used were Rg (medium R of Shepard 1980, as modified by Nelson et al. 1983 with 0.1 g l^{-1} glutamine) and V-KM (Boekelman and Roest 1983 containing 1.0 mg l^{-1} α-naphthaleneacetic acid and 0.4 mg l^{-1} N^6-benzyladenine, personal communication by Mr. D. Foulger, Rothamsted Experimental Station). In order to follow individual heterokaryons (identifiable for one week or more from the combination of chloroplasts of mesophyll protoplasts and cytoplasmic characteristics of suspension protoplasts), fused protoplasts were either embedded in low-gelling-temperature agarose (Sea plaque, 0.4%, Miles Scientific, Stoke Poges, Bucks., UK; or agarose type VII, Sigma Chemical Co., Poole, Dorset, UK) or mechanically isolated by micropipette (Patnaik et al. 1982).

Results

Aggregation and fusion with different electrode arrangements. Using the system of narrowly spaced electrodes, protoplasts were attracted to the electrode wires in the inhomogeneous alternating field and when tightly apposed to one another and to the electrode, they were fused by the pulse. With high protoplast densities, many chains of three or more protoplasts formed and often all of these fused together. At lower protoplast densities, the electrode chamber contained a proportion of chains of only two protoplasts in contact with the electrode. Fusion frequencies of 20–70% were found, but with our equipment the system proved inconvenient for further analysis or culture because of the low numbers of protoplast pairs and the tendency of the protoplasts to stick to the electrode wires.

In the systems using more widely separated electrodes, where mutual dielectrophoresis occurs, the protoplasts were attracted towards each other, but did not move torwards the electrodes. Thus the area of contact of protoplasts may be somewhat less than that in the system of narrowly spaced electrodes, where additional forces are exerted by the divergent field that push the protoplasts against the surface of the wires. An increase of the field strength before application of the fusion pulse did not increase the area of contact, as expressed by the fusion frequency (data not shown). The length of the chains could be influenced by the density of the protoplasts, the strength of the collecting alternating-current field and the duration of the collecting phase.

Pulse duration and voltage – fusion yield relationships. Detailed results are presented only for the experiments with the widely spaced electrodes.

The size of the analytical electrode chamber and the purity of the samples permitted many fusion events to be analyzed rapidly and with good reproducibility (compare Fig. 4) for construction of histograms and pulse duration-fusion response curves. By applying pulses of different duration at different voltages, these characteristics were collected for various types of protoplasts to analyze threshold and maximum values for fusion, and frequencies of single and multiple fusion events in chains.

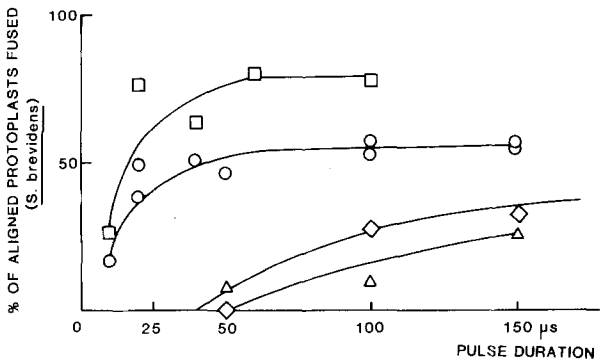


Fig. 1. Pulse duration (µs)-fusion response curves for *Solanum brevidens* leaf protoplasts. The pulse voltage between the electrodes, chain length and sample size of aligned protoplasts were: □, 200 V, >10 protoplasts per chain, 100–200 protoplasts; ○, 200 V, ≦5 protoplasts per chain, 150–170 protoplasts; ◇, 100 V, >10 protoplasts per chain, 160–200 protoplasts; △, 100 V, ≦5 protoplasts per chain, 100–290 protoplasts

The fusion response to increasing pulse duration is presented in Fig. 1 for mesophyll protoplasts of *S. brevidens*. At a pulse voltage of 100 V, the fusion-yield curve intersects the abscissa. This indicates that there is a threshold for fusion. Fusion yield increases with pulse length to reach maximum values of 25% and 55%, respectively, at pulse voltages of 100 and 200 V with a protoplast chain length of ≦5. When the lengths of protoplast chains are allowed to increase (≧ten protoplasts), fusion yields at both voltages are also increased. Damage appears to occur preferentially to protoplasts in long chains.

The percentages of protoplasts taking part in the formation of single, double, triple etc. fusion events, or remaining unfused, are shown in Fig. 2. The results are given for different fusion-pulse durations applied to short and long chains of mesophyll protoplasts of *S. brevidens*. Short pulses applied to short chains do not fuse the majority of protoplasts (Fig. 2a). With longer pulses (Fig. 2b–d) 20–30% of the protoplasts fuse in pairs, but with increasing pulse length more fusion events result in multiple fusion products. Further increase in pulse length leads to protoplast damage. With longer pulse durations (200–300 µs) at lower volt-

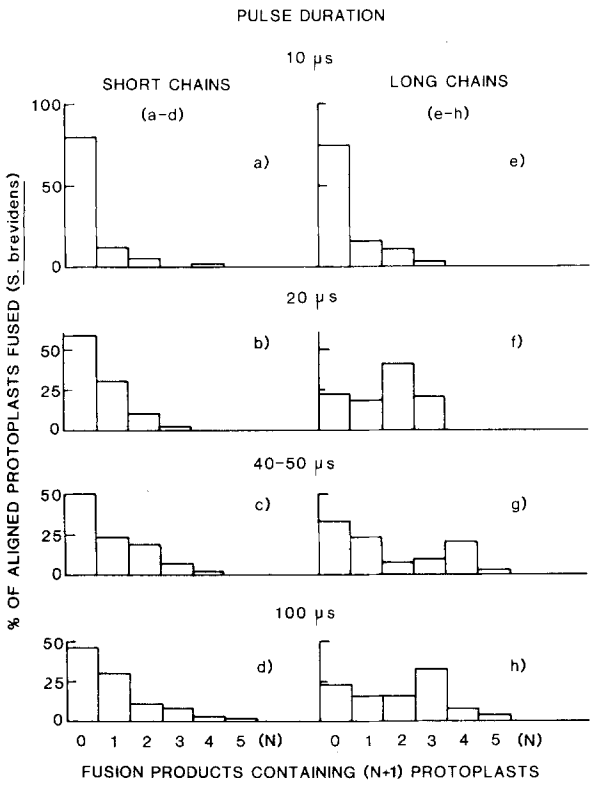


Fig. 2a–h. Analysis of fusion products in long and short protoplast chains (*Solanum brevidens* leaf protoplasts) at different pulse durations. **a–d** Chain length ≤ 5, sample size 330–490 aligned protoplasts. **e–h** Chain length ≥ 10, sample size 80–220. The percentage of aligned protoplasts involved in 0–5 fusion events, yielding unfused protoplasts (class "0") bi- and multikaryons respectively is given. Pulse voltage between electrodes = 200 V

ages, repulsion of protoplast partners, caused by electrophoretic activity, occurred. These effects therefore limit the maximum obtainable values for fusion in pairs to 20–30% of the total protoplasts in chains in this experimental system. When protoplast chains are longer, the probability of more fusion events is theoretically greater, and this is borne out by the results (Fig. 2e–h). Fusion products composed of four and five protoplasts are most frequent at longer durations and higher pulse voltages, and thus the yield of double fusion products tends to be reduced. However, the yields of double fusion products that can be obtained with the long chains is not much lower than that in the case of short chains. In actual numbers, 20–30 of such double fusion products may be obtained from 200–300 associated protoplasts in the analytical fusion chamber.

Characteristics according to protoplast origin and size. The pulse length-fusion relationships of suspension-culture protoplasts of *S. brevidens, N. sylvestris, N. rustica, D. innoxia, T. aestivum* and root-tip protoplasts of *V. faba,* determined at a pulse voltage of 200 V in short chains, are given in Fig. 3. Under these conditions there is a threshold requirement for fusion that pulse lengths must exceed 25–50 µs. With increasing pulse duration there is a rise in fusion frequency, and the maximum fusion values vary with protoplast size (mean protoplast diameters are given in the legend to Fig. 3) as predicted by Zimmermann (1982). The maximum fusion value for the smallest protoplasts, those from *S. brevidens* suspensions (diameter 23 µm), did not exceed 15%, whereas that of the largest protoplasts, of *D. innoxia* (diameter 44 µm), reached a maximum value of 50%.

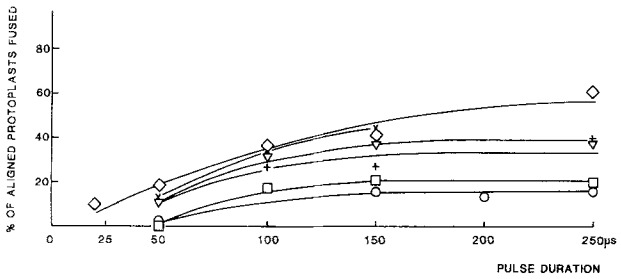


Fig. 3. Pulse duration-fusion response curves for suspension-culture and root-tip protoplasts. The source, mean diameter and sample size of aligned protoplasts were: suspension protoplasts – ◇, *Datura innoxia,* 44 µm, 100–170 protoplasts; ×, *Nicotiana sylvestris,* 30 µm, 100–230 protoplasts; ▽, *N. rustica,* 31 µm, 150–200 protoplasts; +, *Triticum aestivum,* 28 µm, 170–280 Protoplasts; ○, *Solanum brevidens,* 23 µm, 150–200 protoplasts; root-tip protoplasts – □, *Vicia faba,* 29 µm, 110–170 protoplasts. Chain length ≤ 5, pulse voltage between electrodes = 200 V

Although the differences in size of protoplasts of *N. rustica, N. sylvestris, V. faba* and *T. aestivum* were small, differences in maximum fusion yields are evident. This indicates that other factors apart from size should also be considered (see Discussion). The root-tip protoplasts of *V. faba* exhibited similar pulse duration-fusion yield curves to those of suspension-culture protoplasts, but in this case damage to protoplasts occurred when pulse duration exceeded 150 µs.

A response to pulses at 200 V that clearly differs from that of the suspension and root-tip protoplasts (Fig. 3) is evident in Figs. 1 and 4 for mesophyll protoplasts of *S. brevidens* and *T. aestivum.* The threshold for pulse duration is much less than 10 µs. For the larger *T. aestivum* protoplasts (diameter 31 µm), this threshold is so short that at 10 µs the maximum level of fusion has already been reached. However, the maximum fusion yield attained is equal to that for *S. brevidens* (diameter 23 µm). These data indicate a different situation

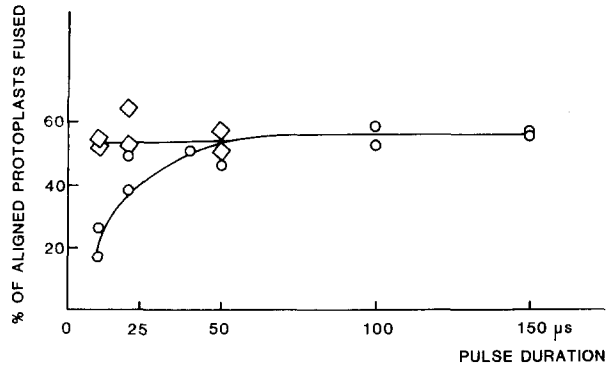


Fig. 4. Pulse duration-fusion response curves for leaf protoplasts. The source, mean diameter and sample size of aligned protoplasts were: ○, *Solanum brevidens*, 23 μm, 150–270 protoplasts; ◇, *Triticum aestivum*, 31 μm, 120–200 protoplasts; other parameters as Fig. 3

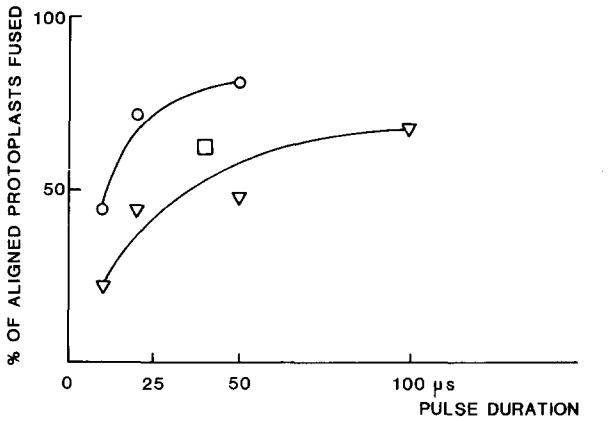


Fig. 5. Pulse duration-fusion response curves, expressed as the percentage of *Solanum brevidens* leaf protoplasts fusing with various suspension-culture protoplasts. The source of suspension-culture protoplasts and number of *S. brevidens* protoplasts were: ○, *Datura innoxia*, 70–80 protoplasts; □, *Triticum aestivum*, 76 (one point); ▽, *Nicotiana sylvestris*, 70–130 protoplasts. Pulse voltage between electrodes = 200 V; chain lengths: *D. innoxia* – long; *T. aestivum* and *N. sylvestris* – short. Low ratio of *S. brevidens:* suspension protoplast mixture

from that depicted in Fig. 3 for the suspension protoplasts. At lower voltages, however, the response curves for *S. brevidens* mesophyll protoplasts (Fig. 1) resemble those of the suspension-culture protoplasts as far as threshold values, response to pulse length and maximum yields are concerned.

The numbers of protoplasts fusing in pairs after alignment in short chains also follows the pattern shown in Fig. 2 for the *S. brevidens* leaf protoplasts, that is, at lower overall fusion levels most fusion products originate from two protoplasts, whilst at higher fusion yields, multi-fusion products become more prominent. *T. aestivum* leaf protoplasts, at 59% overall fusion, have 54% of the fusing protoplasts joined in pairs. This closely matches the 55% value of the *S. brevidens* leaf protoplasts at 54% overall fusion. For *Datura,* wheat and *N. sylvestris* suspension-culture protoplasts, values of 50–75% were recorded at a 40% overall fusion frequency, whilst 86 and 100% values, respectively, were obtained from *V. faba* root-tip protoplasts and *S. brevidens* suspension-culture protoplasts at about 18% overall fusion. When calculated as a percentage of the number of fusion products, this means that 60–100% of the homokaryotic fusion products, generated in chains of about five protoplasts, were the result of fusion between two protoplasts.

Fusion in mixtures of two different types of protoplasts. The results in the previous section indicate that there are differences in response in the electric field that depend mainly on protoplast size, origin (mesophyll; suspension/root-tip), and position in chains.

In a series of subsequent experiments, the effect of protoplast size and origin on fusion was determined for mixtures of different protoplasts.

Extreme difference in size was not a problem, as shown in the successful fusion of leaf protoplasts of *S. brevidens* (diameter 23 μm) with *B. napus* hypocotyl protoplasts (diameter 60 μm) (Fig. 6). The difference in origin also did not pose any problems and leaf protoplasts of *S. brevidens* were fused with suspension-culture protoplasts of *T. aestivum, D. innoxia, N. sylvestris* and *S. brevidens.*

By direct observation of individual fusion events, the fusion frequency was seen to be quite high. Subsequently, experiments to obtain pulse duration-response relationships in heterofusions between leaf protoplasts of *S. brevidens* and suspension-culture protoplasts of *D. innoxia* and *N. sylvestris* were carried out. Low proportions of leaf protoplasts (ratio $<1:10$) were introduced into concentrated samples of suspension-culture protoplasts, and the percentage of the green *S. brevidens* protoplasts that fused to the colourless suspension-culture protoplasts was determined. The experiments were thus designed to give the leaf protoplasts as much exposure to their partners as possible.

The different physical properties of the partners need to be taken into account when evaluating the results. The large *Datura* protoplasts (diameter 44 μm) aligned quickly into long chains under the collecting-field conditions required to aggregate the smaller (23 μm diameter) leaf protoplasts of *S. brevidens.* From their large size, it is clear that considerable forces operate over the rela-

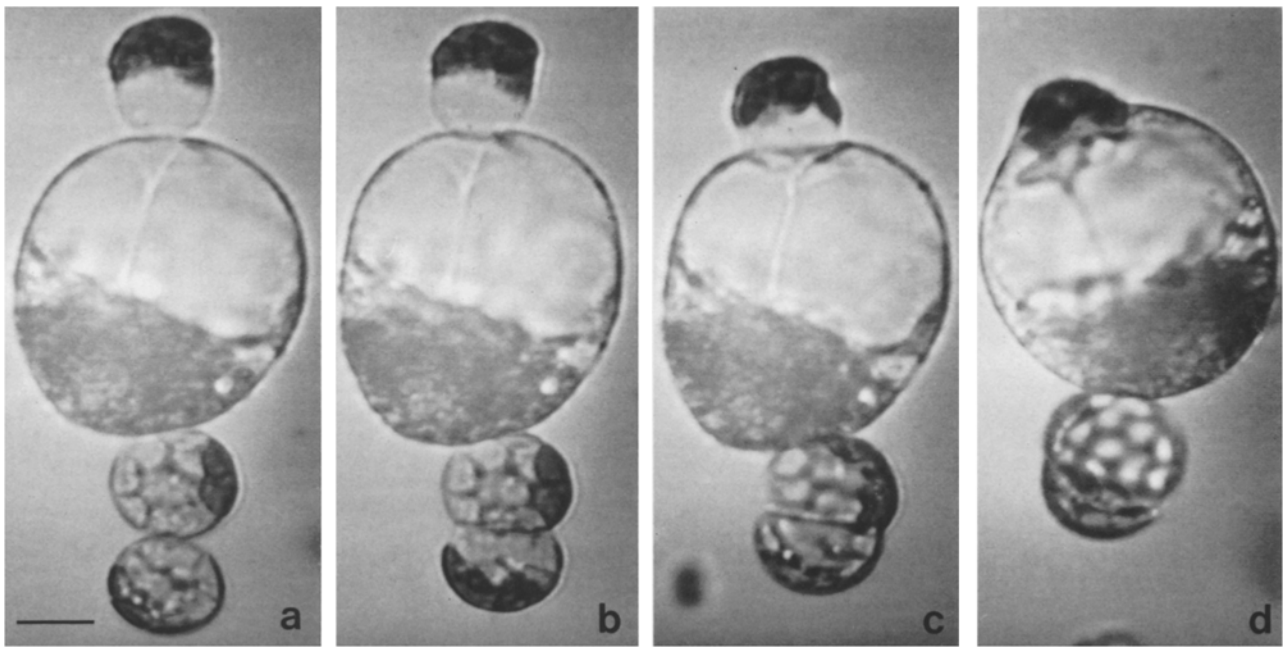


Fig. 6a–d. Fusion of large hypocotyl protoplast (*Brassica napus*) with small mesophyll protoplast of *Solanum brevidens* and homofusion of two mesophyll protoplasts. **a–c** 1–5 min after pulse, **d** 15 min after pulse. Bar = 13 μm. (Photographed from monitor of videorecording of electrofusion)

tively large distances at the poles of the *Datura* protoplasts, and in long chains, forces are such that terminal protoplasts showed signs of cytoplasmic extrusions. The *S. brevidens* protoplasts were pushed from between neighbouring *Datura* protoplasts when the field was maintained for too long.

Under these conditions, the shape of the pulse-response curve for the fusion of protoplasts of *S. brevidens* with protoplasts of *D. innoxia* (Fig. 5) was the same as shown for self-fusion of *S. brevidens* (Fig. 1, long chains). Clearly the limited fusion-response properties of the *Datura* protoplasts under the electrical fusion parameters used did not reduce the fusogenic properties of the *S. brevidens* leaf protoplasts. In fusions of protoplasts of *S. brevidens* with protoplasts of *N. sylvestris,* the initial situation was different. The size of these particular suspension-culture protoplasts (23 μm diameter) was the same as *S. brevidens* leaf protoplasts, but the *N. sylvestris* protoplasts were less mobile in the field (because of their size/density ratio). Consequently, chains were rather short and so the pulse-fusion response curve was like that of *S. brevidens* leaf protoplasts self-fusing in short chains (Fig. 1). One test with *T. aestivum* protoplasts indicated an intermediate situation compared with the previous partners (Fig. 5).

Viability of treated protoplasts. In order to test the viability of protoplasts subjected to electric fields, experiments were carried out in which protoplasts were treated with fluorescein diacetate (FDA) before fusion, in both analytical and large-volume electrode chambers. *T. aestivum* suspension protoplasts treated with FDA are illustrated in Fig. 7, before, during and after fusion (collection field 1 MHz, 8 V; fusion pulse 200 V, 50 μs). Fluorescein accumulated by the protoplasts was retained during chain formation and the fusion pulse, and was detected without noticeable loss 15–30 min afterwards at the same intensity in fusion products, non-fused protoplasts and protoplasts not subjected to the field. With longer pulses, visible damage to protoplasts correlated with loss of fluorescence. It is concluded that under standard fusion conditions protoplast viability as determined by FDA staining is not impaired.

Cell-wall tests. Since suspension-culture protoplasts yielded fewer electrofusion products than leaf protoplasts, they were examined by staining with calcofluor M2R for possible wall formation. This fluorescent stain did not bind to suspension protoplasts indicating that walls were absent and, therefore, that wall formation was not the cause of the lower values for fusion.

Culture of fusion products. To allow identification of heterokaryons, green mesophyll (*S. brevidens*) and cytoplasm-rich colourless suspension-culture

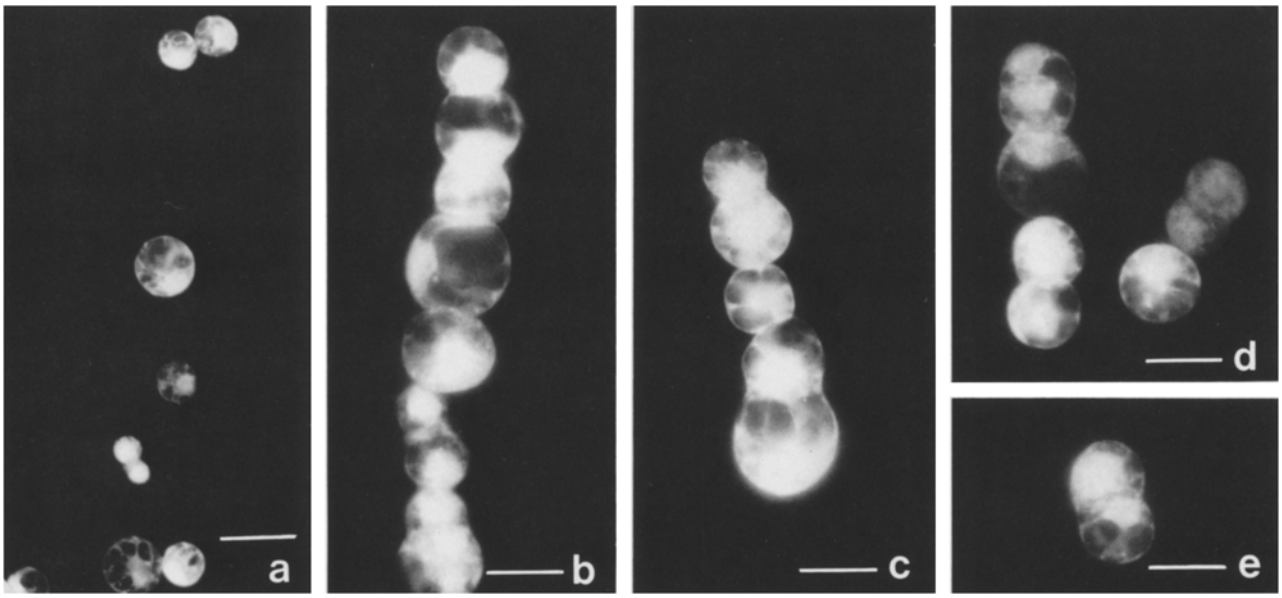


Fig. 7a–e. *Triticum aestivum* suspension-culture protoplasts treated with fluorecein diacetate to test for viability (fluorescein fluorescence) during electrofusion. **a** Protoplasts before application of electric field; **b** chain of nine protoplasts aligned in homogenous field, just after application of fusion pulse; **c** 5 min after fusion pulse two pairs of the five protoplasts have fused; **d, e** fusion products 5–10 min after pulse application. The fluorescence appears to be stable during electrofusion. **a** Bar = 44 μm; **b–d** bar = 22 μm

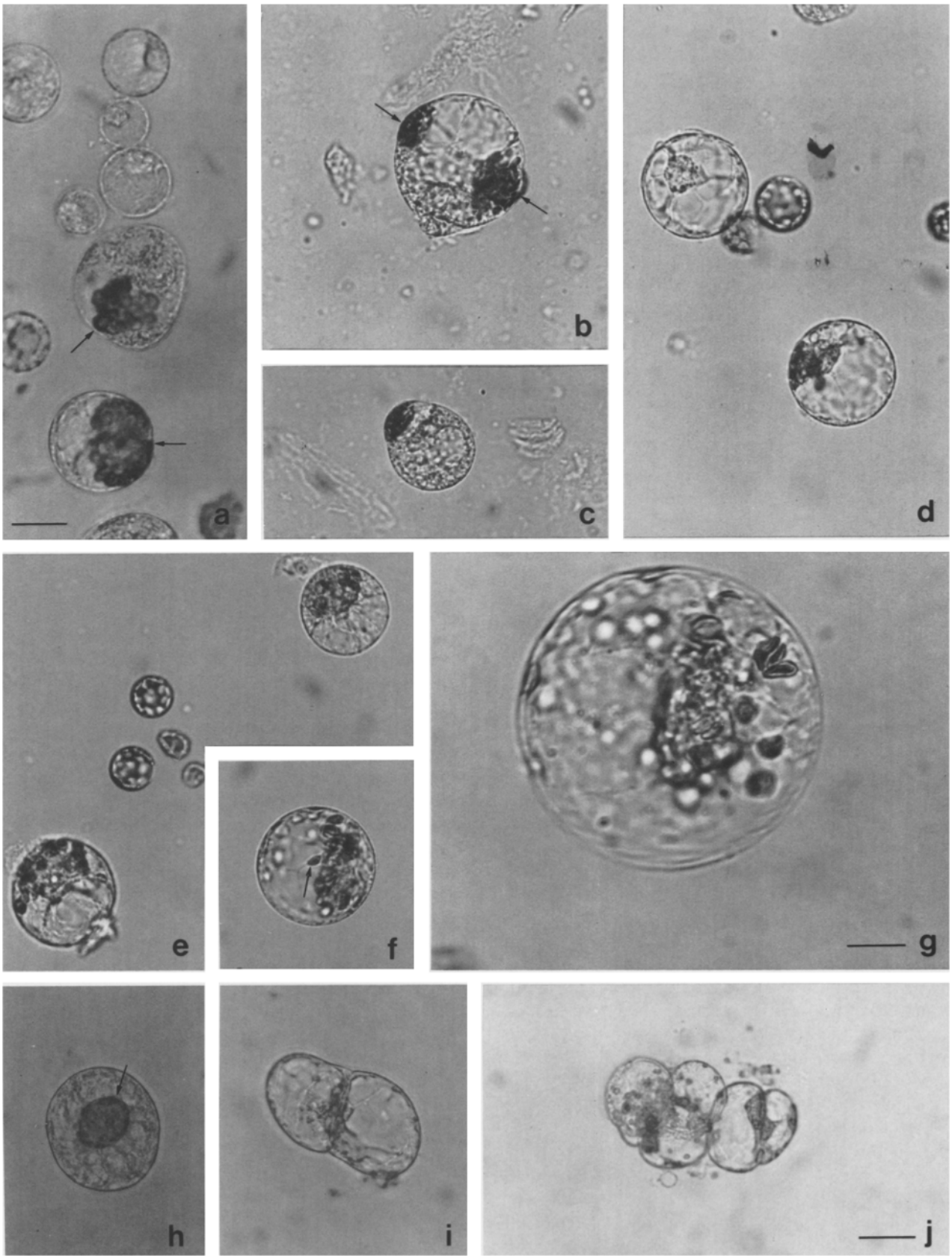
(*N. sylvestris* or *D. innoxia*) protoplasts were fused together. Following electrofusion, the contents of the batch electrode chamber were transferred to a dish with a pasteur pipette. There were no obvious signs of damage to single protoplasts or fusion products at this stage, which indicates the short-term nature of the electrically induced membrane instability (compare Zimmermann 1982).

The products were cultured either in liquid media (V-KM or Rg) or in media solidified with 0.4% agarose. Heterokaryons could be identified for up to 10 d after fusion in both systems, but to ensure unequivocal identification the co-ordinates of heterokaryons embedded in agarose were recorded, such that the development of individual heterokaryons could be followed. The course of development – initial polarised distribution of chloroplasts from mesophyll partners (Fig. 8a–c), their redistribution throughout the cytoplasm (Fig. 8e–g), cell-wall formation and tendency to cluster around the nucleus (36–72 h) (Fig. 8h), and subsequent initial division (Fig. 8i, j) after 4–8 d followed an apparently similar course of events as heterokaryons of the same partners induced by polyethylene glycol-Ca^{2+} at a high pH (personal communication by D. Foulger, N. Fish and S.W.J. Bright Rothamsted Experimental Station). Clearly heterokaryons produced by electrofusion in this way are viable. Further culture has yielded callus and shoot regeneration, and analysis of hybrid material is in progress.

Discussion

Comparison of electrode arrangements. It is clear from the results that the variation we have used of the technique of Zimmermann (1982), that is, application of a collecting field between relatively widely separated electrodes (Richter et al. 1981; Watts and King 1984) is useful for preparative as well as analytical experiments on fusion of plant protoplasts. Although the area of membrane contact between the fusion partners before fusion may not be as great as in the geometrically inhomogeneous field system, fusion is achieved in sufficient

Fig. 8. Heterokaryons of *Solanum brevidens* mesophyll and *Datura innoxia* or (h) *Nicotiana rustica* suspension-culture protoplasts. **a** 5 min after fusion, chloroplasts *arrowed;* **b** fusion between two mesophyll (*arrows*) and one suspension-culture protoplast (10 min); **c** one-to-one fusion (10 min); **d–g** 24 h after fusion; **d** one of each parental protoplast type, one heterokaryon; **e** two heterokaryons and two mesophyll protoplasts; **f** heterokaryon with chloroplast in cytoplasmic strand (*arrow*); **g** heterokaryon, chloroplasts clearly visible; **h** 36 h after fusion, the chloroplasts (*arrow*) of *S. brevidens* have all clustered around the nuclei; **i** 7 d after fusion, dividing heterokaryon; **j** colony in agarose 21 d after fusion. **a–f, h, i** Bar = 22 μm; **g** bar = 11 μm; **j** bar = 44 μm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yields over a range of protoplast densities and aggregation times using simple electrodes and manipulations. Probably, fragile protoplasts like those from leaves are less liable to be damaged in this system because most protoplasts do not contact the electrodes. The possibility of directing the fusion process towards larger-scale one-to-one fusion between different partners, which is theoretically possible but has yet to be reported for plant protoplasts using the system of narrowly spaced electrodes (Zimmermann 1982) is not an obvious feature of the mass-fusion arrangement using widely spaced electrodes, but the exploitation of natural or induced properties of the protoplasts can partly solve this problem (see below).

Analysis of fusion frequencies in chains of similar protoplasts. In principle, if fused products from two partners are desired as is usually the case, it would be logical to try to keep chain length down to two protoplasts. However, this would require either low densities of protoplasts, or with higher concentrations, that the aggregation period should be short and carefully controlled.

In practice, protoplasts aligned in longer chains proved satisfactory for production of fusion products from two partners. In chains of similar protoplasts, the number of fusion events is determined by the voltage and the duration of the fusing pulse. With longer pulses, more fusion events occur and the probability of more than two partners fusing together increases, thus reducing the number of fusion products between two protoplasts. Optimum electrical conditions may be established to generate either a high number of bikaryons in conjunction with multifusion products (multikaryons) or a lower number without much 'contamination' with multifusion products (Fig. 2). Within a chain, probabilities of a protoplast fusing are obviously less for the terminal protoplasts and this is reflected in the higher fusion frequency when chains are longer and consequently terminal protoplasts are proportionally less frequent. However, an additional effect may occur when chain length exceeds 10–20% of the electrode gap. In such cases, damage, or at best, mass fusion may result, as indeed was often observed when protoplast chains bridged the electrodes, and this is more likely to occur if the electrodes are closer together.

When the pulse-fusion response curves were determined for protoplasts obtained after alignment in short chains, the role of size, as predicted by physical laws (Zimmermann 1982), was apparent but not predominent. For the suspension-culture and root-tip protoplasts, and also for the leaf protoplasts, there was, as predicted, an approximate correlation between size and fusion frequency (Figs. 3, 4), the smallest and the largest protoplasts fusing at lowest and highest frequencies, respectively. However, there was some variation in response in the intermediate-size range of the suspension-culture protoplasts which may either be related to different plasma-membrane surface properties or to differences in cytoplasmic distribution internal to the membranes, a feature evident in these cells, but this has not yet been investigated in detail. The dramatic difference in fusion response between suspension-culture and leaf protoplasts is probably based on the different membrane composition and metabolic activities of these two morphologically distinct types.

When comparing protoplasts of equal size from leaves and suspension cultures, for leaf protoplasts, a lower threshold for fusion was evident for the same voltage pulse. The fusion yield for leaf protoplasts increased more sharply with increasing pulse duration to reach a much greater level than that of suspension-culture protoplasts. At lower pulse voltages, the duration-response curve of mesophyll protoplasts resembled that of the suspension-culture protoplasts. This indicates that the initial events of fusion occur more efficiently in the leaf protoplasts. However, for the same pulse lengths applied to leaf protoplasts at 100 V or 200 V, as can be seen from Fig. 1, the same fusion values are not attained. Either the population of leaf protoplasts is not homogeneous, and a subpopulation requiring a higher pulse voltage for fusion is present, or in some way the higher-voltage pulse may open more pores in the membrane of the whole population, thus inducing more efficient fusion than at the lower-voltage pulse.

The results clearly show that the fusion response of protoplasts is governed by physical factors. The most obvious parameters include voltage and duration of the fusion pulse, and protoplast size. Other factors which govern the electrical (fusogenic) response of the membranes include membrane and cytoplasmic properties. The measurement and inter-relations of contributing system parameters such as membrane relaxation time (τ), internal resistance, membrane resistance, capacitance, and breakdown voltage, are discussed at length by Zimmermann et al. (1981), Zimmermann (1982). The properties reflect the origin of the protoplasts: suspension-culture protoplasts are generally considered to be more 'robust' and able to withstand the manipulations, for example, of polyethylene-glycol-induced fusion than mesophyll protoplasts. In electrofusion, these properties lead

to very different optimum fusion conditions for suspension-culture protoplasts.

Fusion between protoplasts with different response to the electric field. In fusions between leaf and suspension-culture protoplasts, the pulse duration-fusion frequency response curve (Fig. 5) clearly indicates that the fusion response is determined by the leaf protoplasts (compare Fig. 1). This means that differences like origin and size need not be an important obstacle in electric fusion, and indeed protoplasts with considerable size differences were fused without obvious damage (Fig. 6). It may thus be envisaged that particles which are very small compared with the protoplasts, such as liposomes or bacterial spheroplasts, may also fuse with protoplasts if conditions are chosen such that fusogenic events are induced in the protoplasts.

A further consequence of these properties is that in mixtures between protoplasts that are more and less sensitive to electrofusion, conditions may be selected to favour fusion between partners of different origins. To this end, the most sensitive protoplasts (e.g. mesophyll protoplasts) may be added in smaller numbers to the more resistant ones (e.g. suspension-culture protoplasts) and the pulse length and voltage kept low enough to induce fusogenic events only in the sensitive protoplasts, so that only these protoplasts will fuse with their neighbouring less-sensitive partners. When combined with a selection system (e.g. complementing mutants, selection by micromanipulation) the formation of homokaryons may not be so important and the ratio of sensitive to insensitive protoplasts may be changed by addition of more sensitive protoplasts to the mixture. In this way, high numbers of heterokaryons may be generated. It may also be possible to influence the fusion characteristics of one of the partners, for instance by modification of the plasma membranes, or by evacuolation.

Culture of electrofused products. As demonstrated in the results, the heterokaryons obtained by electrofusion were viable, and capable of cell-wall formation and division in a similar way to heterokaryons induced by polyethylene gylcol. We have, indeed, regenerated shoots from callus obtained after electrofusion.

The technique of electrofusion clearly holds great promise for directed production of one-to-one heterokaryons from plant protoplasts. The relative simplicity, potential for high fusion frequencies and control over events compared with polyethylene-glycol-induced fusions is evident.

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