QUANTITATIVE EVALUATION OF YIELD AND LYSIS IN ELECTROFUSION OF OAT AND WHEAT MESOPHYLL PROTOPLASTS

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ABSTRACT

Electrofusion was carried out using oat (*Avena sativa* L. cv.Solidar) and wheat (*Triticum aestivum* L.cv. Transilvania) mesophyll protoplasts. The effect of DC square pulse parameters on electrofusion yiled and lysis has been systematically assessed. Optimal DC square pulse parameters could be calculated from the value of membrane polarization time in correlation with 50% protoplast lysis.

Key words: electrofusion yield, electric lysis, oat and wheat protoplasts.

INTRODUCTION

Since the first reports on plant protoplast electrofusion, more than fifteen years ago (Senda et al., 1979); Neumann et al., 1980; Zimmermann and Scheurich, 1981) regeneration of both sterile and fertile somatic hybrids have been reported (Morikawa et al., 1987; Han San et al., 1990) and cell reconstitution has been performed (Spangenberg and Schweiger, 1986). However, little attention has been payed to the heterogeneity of protoplast preparations, types and numbers of protoplast fused, protoplasts and fusion product viability (Saunders et al., 1986; Rákosy-Tican et al., 1988) or quantitative evaluation of electrofusion efficiency as determined by electrical parameters. Electrofusion and electroporation, although very elegant and efficient techniques for genetic manipulation of cells, still remain to some extent empirical approaches, the electrical parameters being chosen on the basis of trial and error. Recently, it has become more and more evident that cell density and direct current (DC) pulse strength and duration have a critical role in determining electrofusion yield and fusion product viability (Rákosy-Tican et al., 1988; Mehrle et al., 1990). A systematic study of efficient RNA transfer by electroporation of tobacco protoplasts depending on pulse type and parameters has been also reported (Saunders et al., 1989). For efficient protoplast membrane electropermeabilization, quantitative relationship between the electrical parameters could be calculated (Joersbo et al., 1990). Via electrofusion techniques the physical membrane properties of plant protoplasts could also be analysed (Lucaciu et al., 1988; Mehrle et al., 1990). Such correlations allow a more accurate optimalization of plant protoplast electrofusion and electroporation.

In this paper we report a quantitative systematic evaluation of oat and wheat mesophyll protoplast electrofusion yield and lysis in response to different DC square pulse voltages and durations. On the basis of these experiments, a correlation between physical membrane properties and electrofusion optimalization is also discussed.

MATERIALS AND METHODS

Plant material

Oat (*Avena sativa* L.cv. Solidar) and wheat (*Triticum aestivum* L.cv. Transilvania) caryopses were germinated on a cheesecloth layer moistened with tap water. They were maintained for the first two days in darkness. Subsequently, the plantlets were grown at 500 lux, 25^0C, and were harvested 8 days after germination.

Protoplast isolation

Protoplasts were isolated from the mesophyll tissue of the first leaf using an enzyme mixture consisting of 3% (w/v). Cellulase Onozuka SS, 0.5% Macerozyme R-10, 10 mM $CaCl_2 \cdot 2H_2O$ and 2 mM ascorbic acid in 0.6 M sorbitol (pH = 5.8) Leaf longitudinal slices, about 1-2 mm wide, were incubated in the enzyme solution at room temperature in darkness

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for 16 h. Protoplasts were harvested by filtration through nylon filter (100 μm) and by centrifugation (100 g, 3 min.) three times in 0.6 M sorbitol. They were then resuspended in 0.6 M sucrose covered by a layer of 0.6 M sorbitol. The two-step gradient was centrifuged (200 g, 5 min.) and protoplasts were colected from the sorbitol layer at the top of the gradient.

Electrofusion device and parameters.

The electric field sequence was generated by an apparatus SPEC-1 constructed in the Institute of Isotopic and Molecular Technology (Cluj-Napoca, Romania). The apparatus can generate programmable sequences of 1 to 9 square pulses with amplitudes varying between 1 and 200 V and lengths in the range of 5 μs – 100 ms. An Orion 1552 oscilloscope was used to monitor the alternating current (AC) field sine wave and DC square wave pulse. The microfusion chamber consists of a microscopic slide with two metallic (aluminium or gold) electrodes which have been vacuum deposited onto the surface. The electrode configuration was obtained by means of microphotolitographic (photoresist) method. The thickness of the electrodes was approximately 1 μm and the distance between the electrodes was 500 μm. The protoplast suspension having an original density of $1x10^6$ protoplasts/ml was pipetted on the surface of the slide to obtain a final density of 40-60 protoplasts between the electrodes in a microscope filed (x 200). Sample were exposed to an AC field of 320 V/cm peak-to-peak amplitude, 750 kHz frequency for 5 s, parameters found to be appropriate for oat and wheat protoplasts Rákosy-Tican et al.,

1988). AC fields were followed by two DC square pulses given at 20 ms intervals. Two pulse sequences having different field voltages and durations were applied. Electrofusion efficiency and protoplast lysis were estimated after each particular electrofusion treatment for at least 100 protoplasts. The electrofusion yield has been calculated using the formula:

$$F = \frac{n_f}{N} \cdot 100 \qquad (1)$$

were: F = the percent of fused protoplasts; n_f = the total number of protoplasts fused (all the protoplasts involved in the electrofusion process, either in pair or multiple fusions have been counted); N = the total number of protoplasts in the electrofusion chamber. Protoplast lysis was estimated before and after each electrical treatment and protoplast damage was calculated by difference using the formula:

$$F = \frac{n_1 - n_0}{N} \cdot 100 \qquad (2)$$

where: L = the percent of lysed protoplasts; n_1 = the number of protoplasts lysed after DC pulse treatment; n_0 = the number of protoplasts lysed which can be detected before DC pulse trearment.

During the experiments, protoplasts were maintained on ice. Three different replicates were perfomed and standard errors were calculated.

RESULTS

A general view on oat (Figure 1) and wheat (Figure 2) mesophyll protoplast electrofusion efficiency as determined by DC square pulse voltages and durations reveals that there
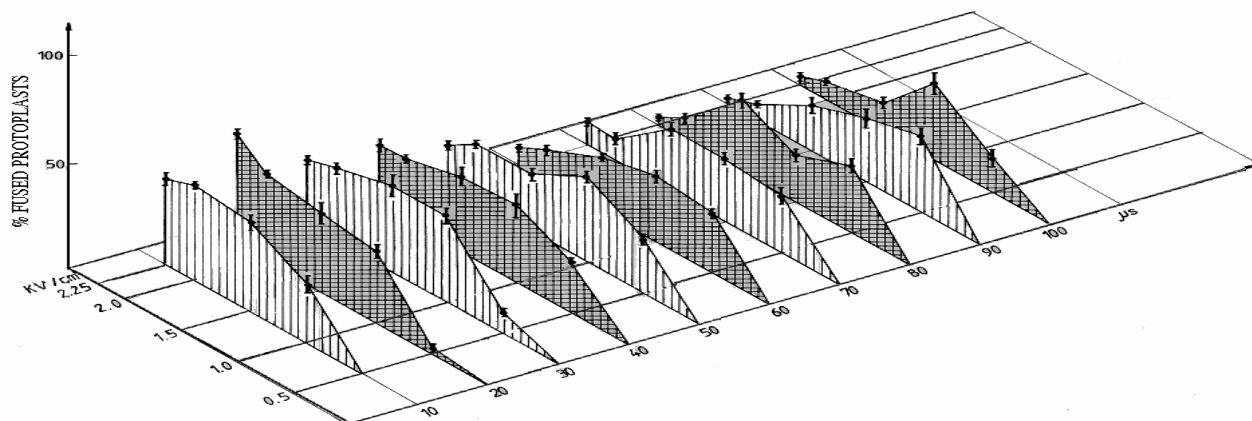

Figure 1. Electrofusion yields (%) of oat (*Avena sativa* L. cv. Solidar) mesophyll protoplasts as determined by DC square pulse voltages and durations. Protoplast density was: 40-60 protoplasts between the electrodes in a microscopic field (x 200). AC field parameters: 320 V/cm, 750 kHz, 5 sec. Two DC pulses have been applied at 20 ms interval. Bars represents standard errors calculated for three replicates
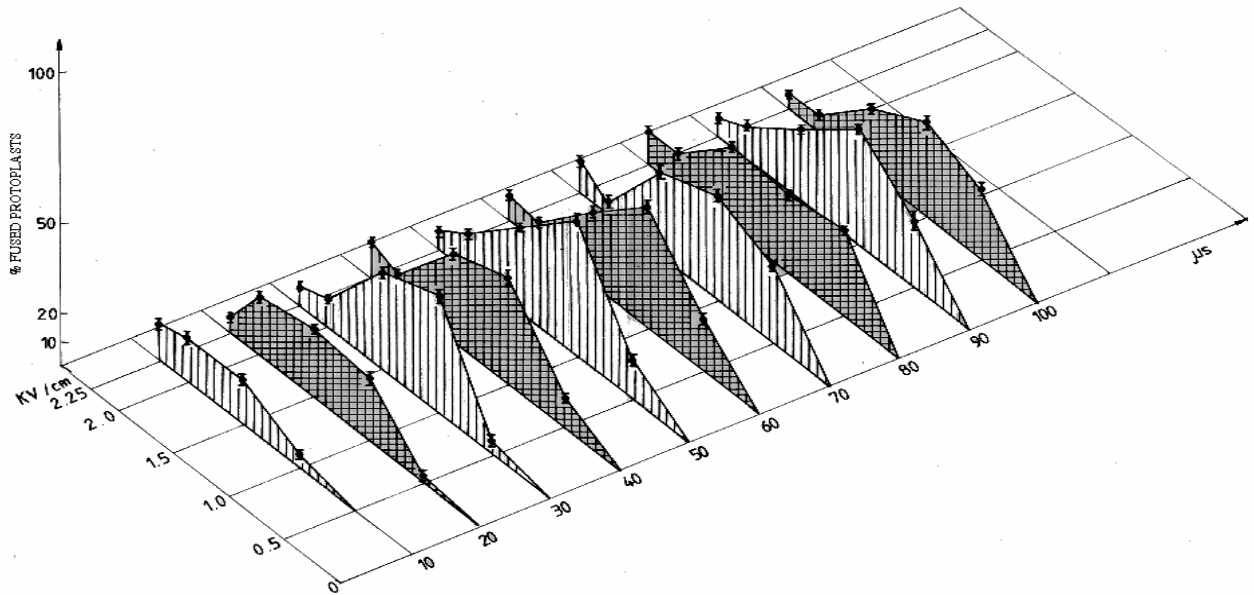
Figure 2. Electrofusion yields (%) of wheat (*Triticum aestivum* L. cv. Transilvania) mesophyll protoplasts as determined by DC square pulse voltages and durations. For other explanations see figure 1.

are differences between the two types of protoplasts investigated. For oat mesophyll protoplasts, the highest electrofusion yields were obtained using short DC square pulses (10-50 μs). The electrofusion efficiency increased with increasing DC pulse voltages (Figure 1). Wheat mesophyll protoplasts were fused with the highest efficiency using DC square pulses of 30 to 70 μs and a field amplitude (peak-to-peak) in the range of 1.0 to 1.5 kV/cm (Figure 2). The range of optimal electrical parameters was also influenced by the lysis of protoplasts. Oat mesophyll protoplasts exhibited a high level of damage with increasing DC pulse voltage and duration (Figure 3). Protoplast lysis exceeded 50% already for DC pulses having 50 μs length and 1.5 kV/cm (peak-to-peak) amplitude. For wheat meso-
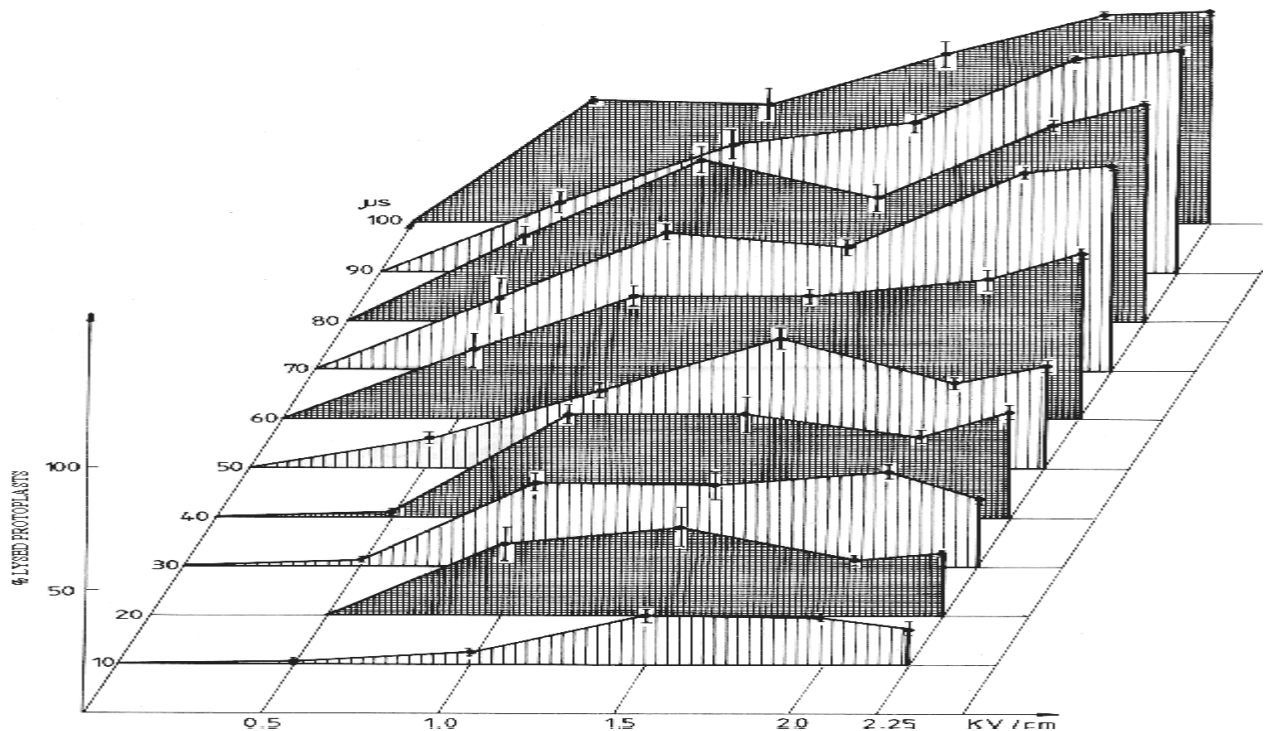

Figure 3. Electric lysis (%) of oat (*Avena sativa* cv. Solidar) mesophyll protoplasts as determined by DC square pulse voltages and durations. For othed explanations see figure 1
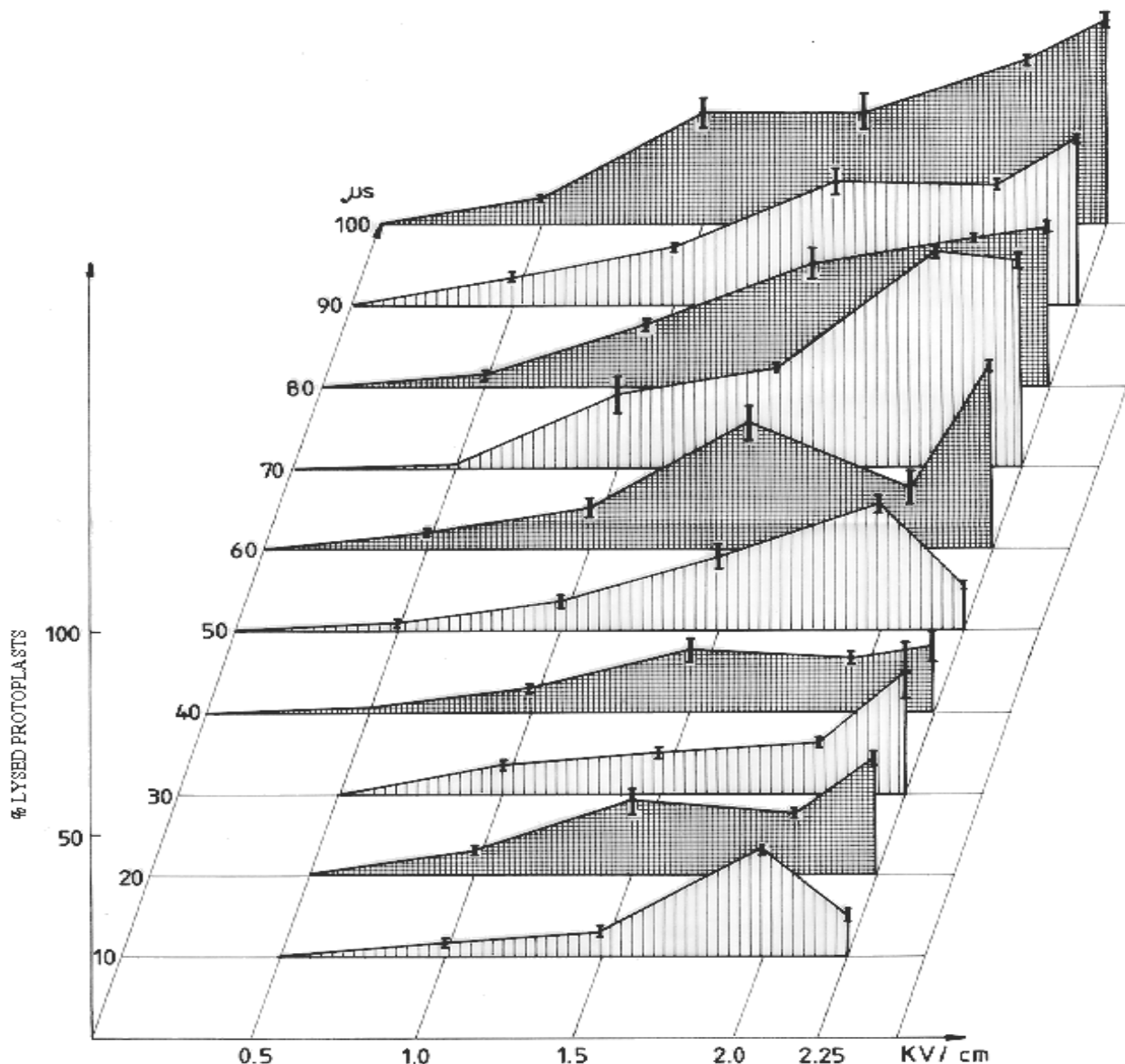
Figure 4. Electric lysis (%) of wheat (*Triticum aestivum* L. cv. Transilvania) mesophyll protoplasts as determined by DC square pulse voltage and durations. For other explanations see figure 1

phyll protoplasts lysis was not as significant (Figure 4). Only an increase of DC pulse length over 70 μs damaged 50% of the protoplasts when the field amplitudes (peak-to-peak) exceeded 2 kV/cm. The membrane polarization time (r) for oat and wheat mesophyll protoplasts were determined in a previous study (Lucaciu et al., 1988), by fitting experimetal data for fusion threshold values with theoretical curves. The experimental curves as presented in figure 5 have been drawn using the values of DC pulse length – for a given DC pulse voltage – for which the first fusion event has occurred in the electrofusion chamber.

That is, the entire population of mesophyll protoplasts has been considered. The dependence on pulse duration of the electric field pulse amplitude which triggers the fusion was given by the equation:

$$E_0 = \frac{2}{3a} \cdot \frac{U_c}{1 - e^{\frac{-t_0}{\tau}}} \qquad (3)$$

were: a = the protoploast radius – the mean value of the protoplast population has been calculated around 15 μm for both species;

U_c = the critical voltage; t_0 = the pulse length; τ = the membrane polarization time constant.

Equation (3) and the critical voltage value from the plateau domain have been used for fitting experimetal data with theoretical curves. From the best fitting we have obtained the polarization time constant for the two protoplast species, i.e. 17 µs for out and 23 µs for wheat mesophyll protoplasts.

The quantitative evaluations on electrofusion efficiency and protoplast lysis could be correlated with the membrane polarization times. Oat mesophyll protoplasts, having a lower membrane polarization time were efficiently fused by shorter DC pulses. When the DC pulse length was more than 3 times higher than the value of membrane polarization time (17 µs x 3 = 51 µs), more than 50% of the protoplast population was damaged. The same correlation was found for wheat mesophyll protoplasts. The pulse length for which the increase of the amplitude get around 50% damage of protoplasts is 3 times higher than the membane polarization time (23 µs x 3 = 69 µs). The membrane polarization time is a value from which the optimal DC pulse length can be calculated. For the optimal range of square pulse duration, the increase of square pulse voltages improved the electrofusion efficiency for both species. Protoplast damage has also increased with increasing pulse voltages, but under 3r the lysis has not exceeded half of protoplast population.

DISCUSSIONS

Plant protoplast electrofusion is a very complex process in which multiple factors are involved. Type of protoplast, enzyme treatment – both affecting the membrane properties – protoplast population density and homogeneity, AC field and DC pulse electrical parameters, all could play an important role. Moreover, homogeneous or heterogeneous, pair or multiple fusions can also occur. For these reasons a very systematic and complete investigation is very difficult to perform. In the literature only data concerning each factor separately has been reported. For instance, the physical properties of plant protoplast mem-

brane proved to influence the electrofusion efficiency in the case of tobacco and oat, vacuolated and evacuolated protoplasts (Mehrle et al., 1990). Membrane polarization time seems to be one of the most important membrane properties for electrofusion. Comparing oat mesophyll protoplast electrofusion threshold values derived from our experiments (Lucaciu et al., 1988) (Figure 5) with the data derived from individual fusions (Mehrle et al., 1990), one could find out that for definite type of protoplast similar values for the membrane biophysical parameters can be obtained.
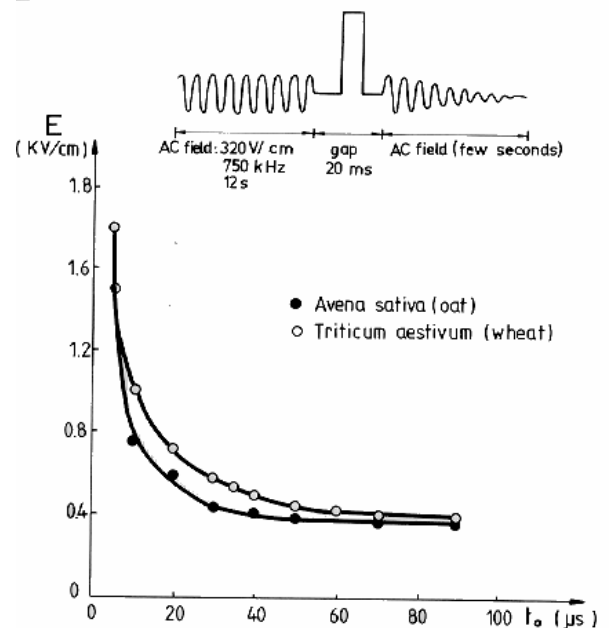

Figure 5. Experimental dependence of the fusion threshold values of DC square pulse length on pulse length in wheat and oat protoplasts

Protoplast density is also an important factor for electrofusion optimalization. The density of protoplasts in the electrofusion chamber should be adjusted to an optimal value. By increasing oat protoplast density, electrofusion yields as high as 98-99% could be obtained (data not shown) using DC square pulses of 30 to 40 µs length and 2 kV/cm strength, but after a few minutes many of the fusion products were lysed. Our microscopical observations revealed that when one protoplast in the pearl chain starts to burst, the next protoplast involved will also be soon disrupted. It is like a chain reaction which finally involves all protoplasts in the pearl chain. This process is obviously more extensive in a dense proto-

plast population. Diheterokaryon formation can be selectively induced by adjusting the density of protoplasts and the AC field parameters (particularly duration and voltage). Optimal protoplast density for tobacco total and dual fusion was in the range of 10^5 - 10^6 protoplasts/ml (Hibi et al., 1988). In our experiments we found out that optimal protoplast density is 40-60 protoplasts between the electrodes in a microscope field (x 200), starting from an original density of protoplast suspension of 10^6 protoplasts/ml.

The effect of AC field parameters on electrofusion efficiency and protoplast viability has been less investigated. AC field frequency in the range of 1 Hz – 8 MHz reduced tobacco protoplast viability only at very low or very high values (Saunders et al., 1986). The range of 20 Hz – 1 MHz had no effect on wheat mesophyll protoplast viability (Rákosy-Tican et al., 1988). *Brassica* protoplasts, however, lysed at 40 V/cm for all sine wave frequencies below 500 kHz (Zachrisson and Bornman, 1984). Exposure time to AC field did not seem to influence tobacco dual fusion (Hibi et al., 1988) or wheat mesophyll protoplast viability. By increasing AC field amplitude the viability of both wheat protoplasts and homokaryons has decreased (Rákosy-Tican et al., 1988). The increase of DC square pulse voltages had detrimental effect on wheat mesophyll protoplasts and homokaryons viability (Rákosy-Tican et al., 1988) and brought about an increase in ethane production in *Vicia faba* protoplasts (Biedinger et al., 1990). Two pulses were found to be optimal for both species (Rákosy-Tican et al., 1988; Biedinger et al., 1990), but survival of tobacco protopoasts has not decreased by increasing the number of pulses up to 10 (Hibi et al., 1988).

As shown in the introduction, plant protoplast density and DC square pulse parameters proved to play a critical role in determining the electrofusion yield and fusion product viability (Rákosy-Tican et al., 1988; Mehrle et al., 1990). In this paper, using oat and wheat mesophyll protoplasts like model systems, in order to screen out the effect of DC square pulse voltages and durations on electrofusion yiled and lysis, we maintained all the other

factors constant. Both protoplasts have been isolated from leaves having the same age, grown under similar conditions, using the same enzyme treatment and washing protocol. The density of the protoplast population has been maintained constant. The AC field frequency and amplitude used in these experiments proved to be the best in preserving wheat mesophyll protoplast and homokaryon viability, as evidenced by flouresceine diacetat (FDA) test (Rákosy-Tican et al., 1988). Two pulses have been applied, a number which gave good results for both species and did not affect the viability. However, differences between the two types of protoplasts still exist, such as the efficiency of protoplast isolation, the effect of the enzyme treatment on protoplast membrane or differences in the dielectrophoretical alignment of the protoplst. The membrane polarization time should be not influenced by the enzyme treatment since similar values could be obtained from different experimental conditions (see above). On the other hand, the dielectrophoretical alignment can modify the quantitative values, but not the general evaluation of the process. One should expect that for a particlular AC field, similar curves should be drawn.

Our results show that for a particular AC field treatment a correlation between electrofusion optimal DC square pulse parameters with the membrane polarization time and 50% protoplast lysis can be established. Such a correlation should be valid for other protoplast types as well.

Acknowledgements

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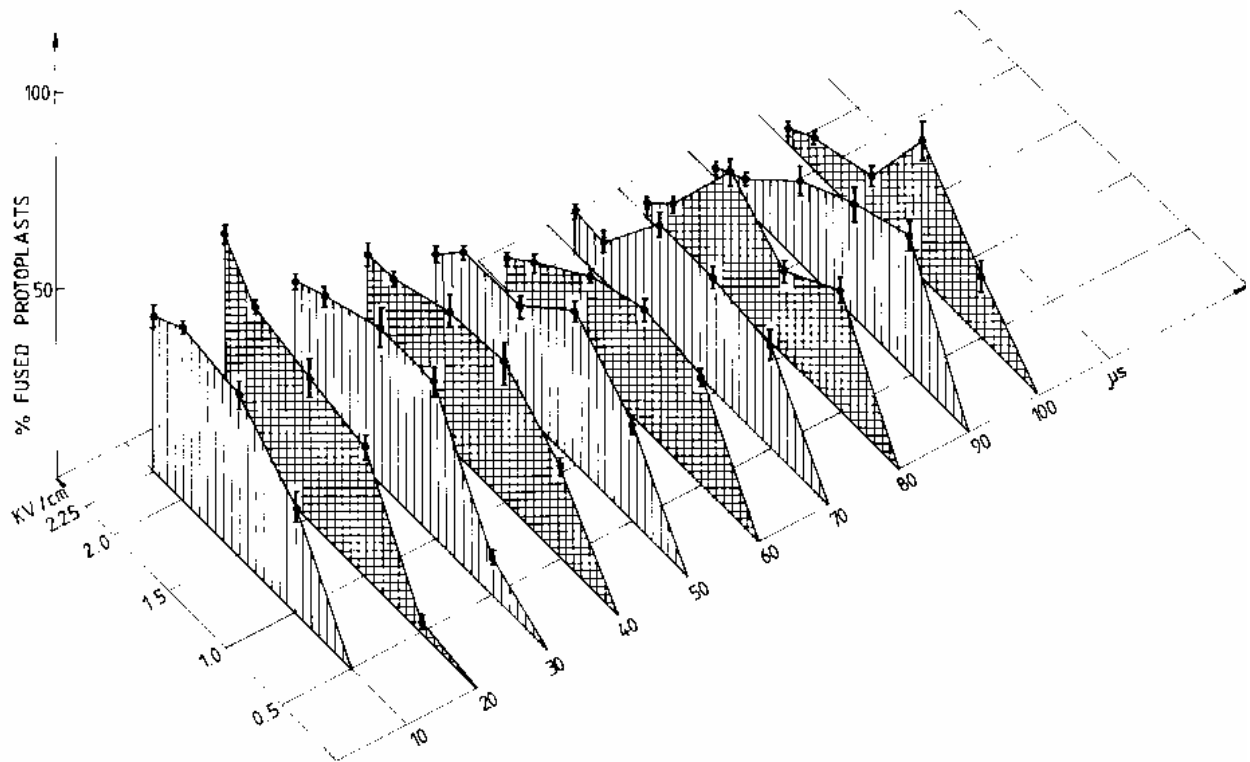
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Figure 1. Electrofusion yields (%) of oat (*Avena sativa* L. cv. Solidar) mesophyll protoplasts as determined by DC square pulse voltages and durations. Protoplast density was: 40-60 protoplasts between the electrodes in a microscopic field (x 200). AC field parameters: 320 V/cm, 750 KHz, 5 sec. Two DC pulses have been applied at 20 ms interval. Bars represents standard errors calculated for three replicates.
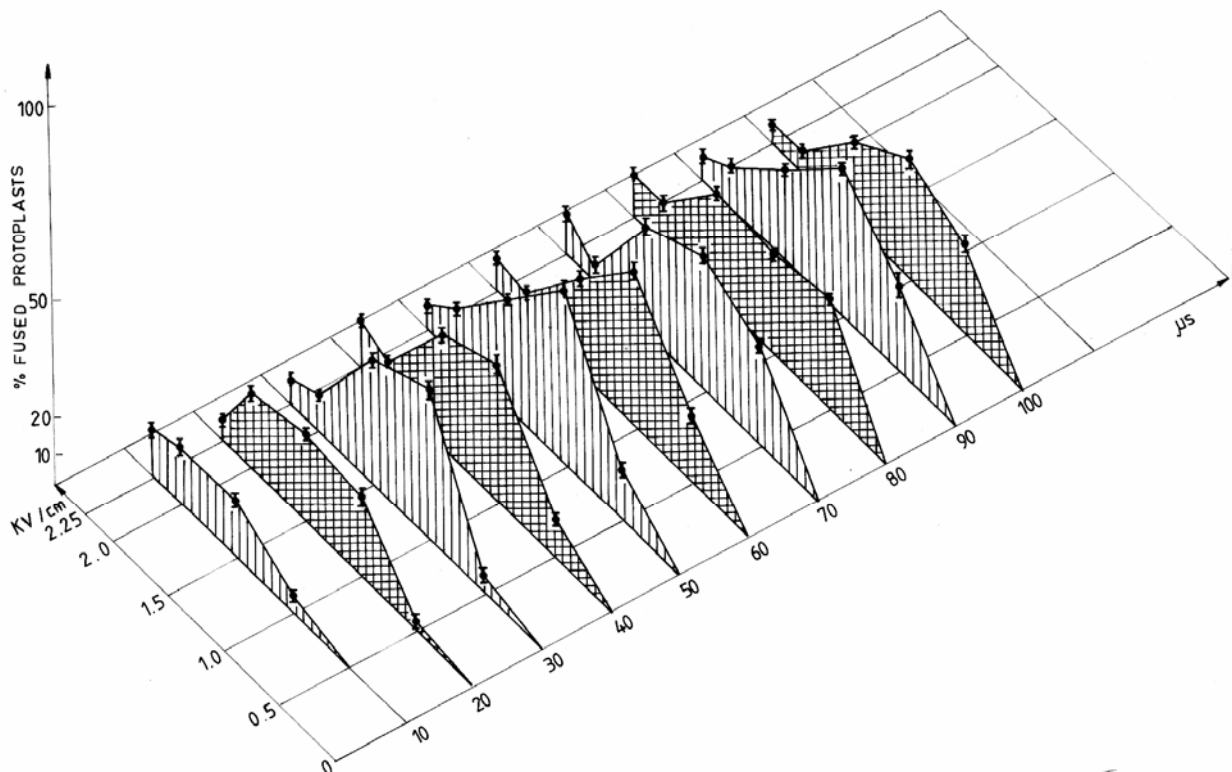

Figure 2. Electrofusion yields (%) of wheat (*Triticum aestivum* L. cv. Transilvania) mesophyll protoplasts as determined by DC square pulse voltages and durations. For other explanations see figure 1.
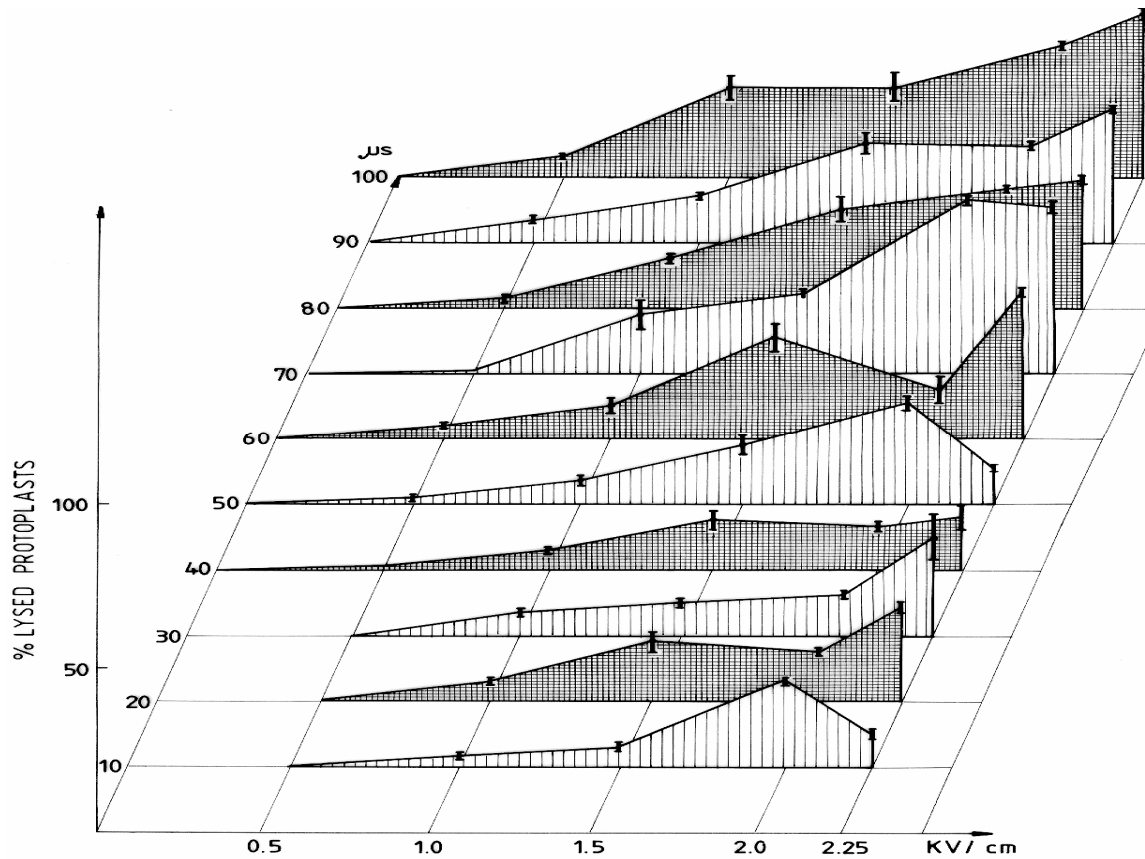
Figure 3. Electric lysis (%) of oat (*Avena sativa* cv. Solidar) mesophyll protoplasts as determined by DC square pulse voltages and durations. For othed explanations see Figure 1.
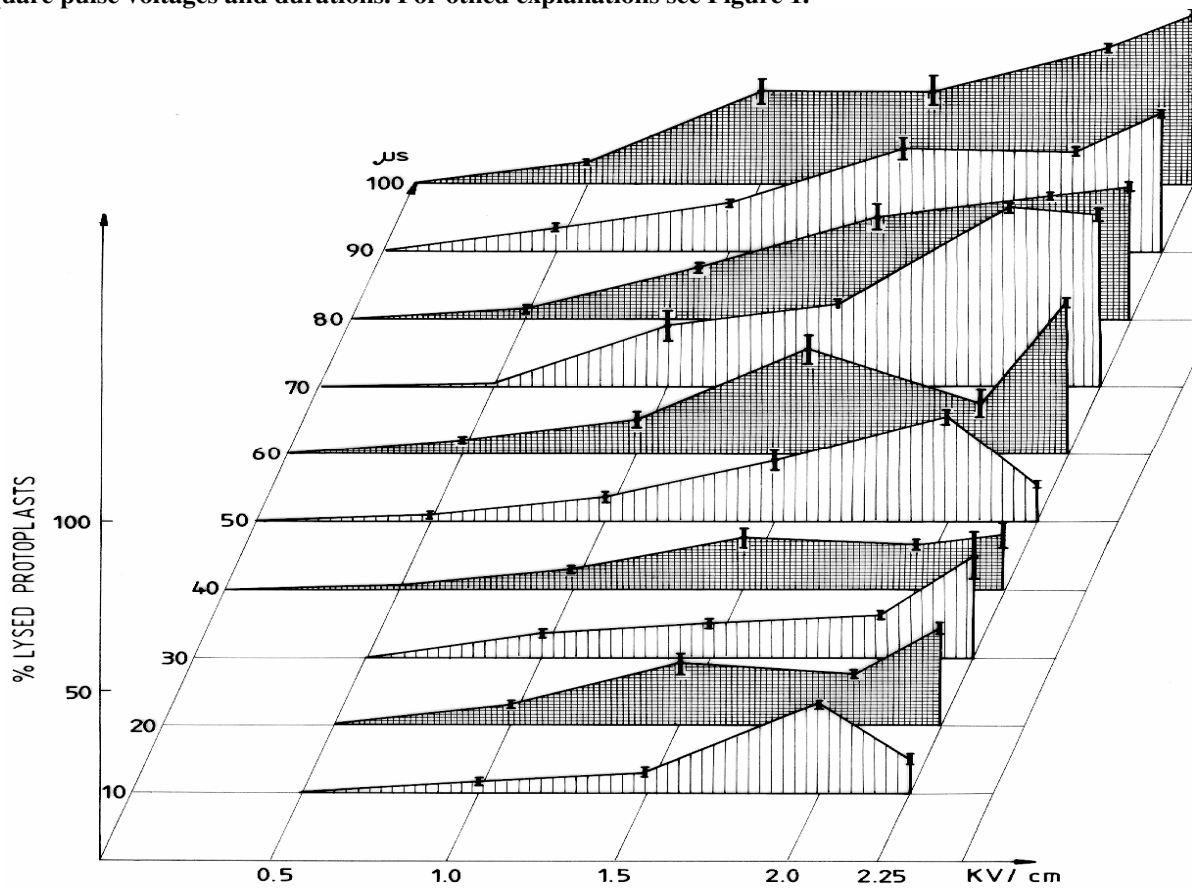

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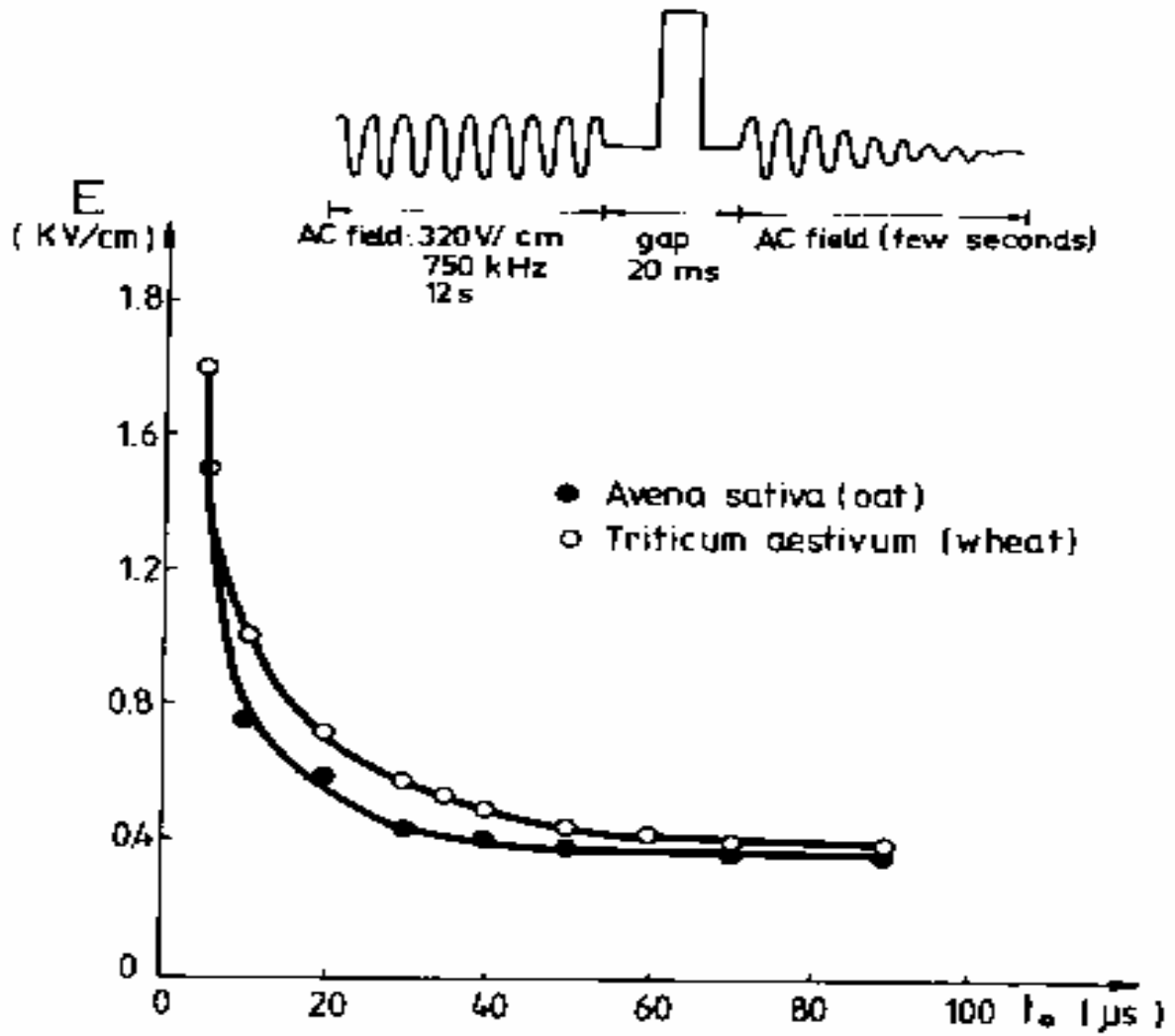
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Figure 5. Experimental dependence of the fusion threshold values of DC square pulse lenght on pulse lenght in wheat and oat protoplasts.

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