Theor Appl Genet (1986) 72:279–286

Fusion of plant protoplasts: a study using auxotrophic mutants of *Nicotiana plumbaginifolia*, Viviani

I. Negrutiu *, D. De Brouwer, J. W. Watts **, V. I. Sidorov *, R. Dirks and M. Jacobs**

Institute of Molecular Biology, B-1640 St. Genesius-Rode, Belgium

Received November 20, 1985; Accepted December 15, 1985
Communicated by Yu. Gleba

Summary. Protoplast fusion studies between various auxotrophic mutants of *Nicotiana plumbaginifolia* were performed to optimize conditions for PEG-mediated fusion and to identify factors influencing the plant protoplast fusion process. Numerous parameters in the isolation, culture, and fusion of protoplasts were tested, and established fusion protocols were compared. Fusion rates, calculated on the basis of colony growth on selection medium (genetic complementation), ranged from 10^{-4} to 10^{-2}. Conditions that allow rapid and reproducible fusions at the highest rates were established. Particular emphasis was given to fusion of mesophyll-derived protoplasts, for which the ability to regenerate fertile plants from fusion products was shown to be particularly high. Preliminary experiments using electric-field mediated fusion suggest that electrofusion may offer significant advantages over the traditional chemical fusion.

Key words: Protoplast – *Nicotiana plumbaginifolia* – Auxotrophs – Electrofusion – PEG

Introduction

Fusion methods with plant cells have been devised empirically since the early 70's and many of the available procedures are in use as modifications of already modified original ones. However, little insight has been made in the fusion events per se. Only two reports (Kao and Michayluk 1974; Kao et al. 1974) analyse various fusion parameters of what is known as the "Kao method". In other papers, the effect on fusion efficiency of different fusogens (Hauptmann et al. 1983), of concavaline A (Glimelius et al. 1978), of the cell cycle stage (Ashmore and Gould 1982), and of both Ca^{2+} salts and sugar content in the PEG solution versus the protoplast source and high Ca^{2+}/high pH treatment (Berry 1983) were investigated. Fusion rates were most frequently estimated by cytological/microscopic methods (per cent heterokaryon formation), the rates being within the range of 10^{-1} (see Gleba and Sytnik 1984). When the rates were measured by genetic methods using selectable markers (Glimelius et al. 1978; White and Vasil 1979; Harms et al. 1981; Menozel et al. 1982; Hauptmann et al. 1983; Negrutiu et al. 1985), they ranged between 10^{-2} to 10^{-6}, with the lowest values for mesophyll-mesophyll protoplast fusions. Rates of 18% were reported by Menczel et al. (1982) when a cytoplasmic marker was employed to select for fusion products.

Electric field-induced cell fusion is being developed (Zimmermann and Schurig 1982; Zimmermann 1982). Commercial equipment has high costs, and very small volumes of cells (4–100 µl; also see Bates and Hasenkampf 1985) are usually fused at the same time. Watts and King (1984) have developed transferrable electrodes that can accommodate 1 ml volumes of protoplasts which were shown to fuse at high rates.

In this paper we describe an analysis of several parameters involved in chemical fusion, and compare the PEG-procedure with the electrofusion technique. For this purpose, nutritionally deficient mutants of *N. plumbaginifolia* (Negrutiu et al. 1983, 1985) were used as fusion partners, and metabolic complementa-

Present addresses:
* Friedrich-Miescher Institute, P.O. Box 2453, CH-4002 Basel, Switzerland
** John Innes Institute, Colney Lane, Norwich NR4 571, England
*** Institute of Botany, Academy of Sciences of the Ukranian SSR, Kiev, USSR

tion in callus clones derived from fusion samples served to measure fusion efficiency, i.e. the fraction of the fused protoplast population that survived the treatment and stably expressed the wild type phenotype. Fusion products were regenerated into plants and analysed biochemically and/or genetically.

Materials and methods

Protoplast preparation and fusion

Various mutants requiring reduced nitrogen (nitrate reductase deficient, NR^-; Negrutiu et al. 1983; Marton et al. 1982), or amino acids (his^-, met^-, try^-, ile^-; Negrutiu et al. 1985) served as the source of protoplasts. They were isolated from leaves of in vitro propagated cuttings or from callus cultures (Negrutiu 1981; Maliga 1982) by overnight digestion followed by purification on sucrose and washed once in either mannitol, sucrose (15 mM $CaCl_2 \cdot 2\,H_2O$ and 0.4–0.5 M sugar), or W_5 salt (154 mM NaCl, 125 mM $CaCl_2 \cdot 2\,H_2O$, 5 mM KCl, 5 mM glucose) solutions. The protoplasts were resuspended in the wash medium at a final density of 10^6 protoplasts per ml (unless otherwise stated), mixed in a 1 : 1 ratio, and either fused immediately, or stored at 6–8 °C for 2 to 6 h before fusion.

Fusions were performed according to Hein et al. (1983); Kao (1982); Menczel et al. (1981); Power et al. (1976) and are summarized in Table 1. An additional procedure developed by us (CMS protocol) is also presented.

The electrofusion procedure was as reported by Watts and King (1984). It makes use of transferrable electrodes that accommodate 1 ml volumes of culture every 20 s at average densities of 10^5 protoplasts per ml. The protoplasts were aggregated in a radio frequency field (10 V RMS, 0.5 MHz) for 30 to 60 s (chains of 5 to 10 protoplasts) with an interelectrode distance of 5 mm, and fused with variable DC pulses. One hour after fusion, the 1 ml samples were diluted 5 to 10-fold with culture medium.

Control plates consisted of mixtures of fusion partners (no fusion treatment), and exclusively homofusion experiments were also performed.

Selection for metabolic complementation fusion products

All samples were incubated in media supplemented with one of the required amino acids in the case of amino acid auxotrophs, and with $(NH_4)_2$ succinate for NR^- mutants (Negrutiu et al. 1983, 1985). Survival rates, estimated at the 1–2 division stage, are defined as the proportion of dividing protoplasts within the starting protoplast population. The colonies were then sedimented in the Petri dish, washed once with selection medium, and resuspended to a final density of 2,000 colonies per ml in the same medium (devoid of either amino acids, e.g. MDs, or with nitrate as sole source of nitrogen, e.g. MDn; see above and Negrutiu et al. 1983). Growing green calli were counted 3 weeks later, and confirmed as fusion products by an additional passage on minimal solid medium. Fusion rates were calculated as complementation rates:

$$\frac{\text{Number of calli on minimal medium}}{\text{Total number of colonies undergoing selection}}$$

and given as $n \times 10^{-3}$.

Table 1. Composition of basic solutions, timing, and sequence of treatments in several fusion protocols

Fusion procedure and code	Incubation solution before fusion	Substrate and sedimentation time	Density ($\times 10^6$)	PEG solution, final concentraton, and time	High Ca^{2+}/ high pH treatment	Washing procedure
C (Power et al. 1976)	0.4 M mannitol, MS macro, $CaCl_2$ 15 mM	Centrifuge tubes, 80 g, 10 min	0.2	25% PEG 6,000, 4% sucrose, 10 mM $CaCl_2$, pH 5.6; 20%; 15 min	–	Gradual dilution with cult. med. or CPW/Ca and centrifugation
S (Hein et al. 1983)	80% sea water[a]	Plastic Petri dish, 10 min	1	25% PEG 6,000, 0.1 M $Ca(NO_3)_2$, 0.45 M mannitol, pH 9; 18%; 15 min	–	0.275 M $Ca(NO_3)_2$, pH 6, 100 g centrifugation
M (Menczel et al. 1982)	W5	Plastic Petri dish, 20 min	1–4	30% PEG 4,000, 0.3 M glucose, 66 mM $CaCl_2$, pH 5.7; 15%; 10–15 min	Yes	2 washes with culture medium
K (Kao 1982)	0.4 M mannitol, 15 mM $CaCl_2$	Cover glass 20 min	1	50% PEG 1,540, 0.1 M $CaCl_2$, 5mM KH_2PO_4, pH 5.7; 37.5%; 10–15 min	Optional	Direct dilution with culture medium
CMS (this paper)	W5	Plastic Petri dish, 20 min	1–2	40% PEG 4,000, 0.1 M $Ca(NO_3)_2$, 0.4 M mannitol, pH 9; 20–26%; 10–15 min	–	1× wash with W5

[a] Sea water can be replaced with W5 salt solution

Spontaneous mutation rates for the mutant lines used are given below: nia $26:4\times10^{-7}$; nia $8:1.4\times10^{-6}$; cnx $103:3.8\times10^{-7}$; cnx 20, try$^-$, ile$^-$: no revertants; his$^-$: 2×10^{-7}; met$^-$: 5×10^{-4}

With one exception (nia 8 + met$^-$ (one revertant)) no such revertants were seen in control plates during the fusion experiments.

Plant regeneration, biochemical and genetic analysis

Colonies selected from both PEG- and electrofused samples were induced to regenerate (Installe et al. 1985). The regenerants exhibited the expected tetraploid phenotype. Although not systematically checked, it appeared that those regenerated following PEG-fusion had aberrant phenotypes more frequently than those coming from the electrofused batches. Seeds were produced by selfing and the R1 generation was analysed for segregation of NR$^-$, his$^-$, and/or met$^-$ traits. Only NR$^-$ segregants were observed, which is similar to results reported and discussed elsewhere (Negrutiu et al. 1985).

Measurements of nitrate reductase in calli or plants produced in several fusion experiments were also performed. The activity found was lower than in the controls, ranging from 30 to 80% (Dirks et al. 1985).

Results

Fusions were performed according to protocols detailed in Table 1. Fusion efficiency was estimated by taking into account both fusion rates (FR) and the relative number (RN) of fusion products per 10^5 fused protoplasts. Fusion products were obtained with most of the tested procedures, with the exception of treatments including a high Ca^{2+}/high pH step (data not shown). Yields were generally low (FR of $0.07-1.2\times10^{-3}$), and so was the reproducibility of the experiments. In general, the very low survival of protoplasts following the fusion treatment seemed to be the limiting factor.

At that stage, the method designated K appeared the most appropriate for further studies, the protocol being also easily adaptable to electrofusion conditions (see below).

1 Physiological and cultural parameters affecting protoplast condition pre- and postfusion

Hormonal treatments, density effects, and composition of the washing solution were tested. The results are given in Table 2. Thus, addition of hormones during the enzymatic digestion of the tissues, combined with an increase of the initial number of protoplasts to be fused in a dish (up to $5-7.5\times10^5$/10 ml petri dish) and a reduction of hormone concentrations in the culture medium, resulted in higher survival and plating efficiencies, and consequently higher RNs and improved reproducibility in fusion experiments.

Futhermore, protoplasts washed and resuspended in W5 salts fused more readily than those incubated with mannitol or sucrose (Table 2). In W5, the protoplasts sediment rapidly and attach firmly to the base of the dish, resulting in a higher local density and intercellular contact area during PEG agglutination.

Table 2. Effect on fusion efficiency of hormone pretreatment, initial amount of protoplasts to be fused, hormone concentration in the culture medium, an sugar and/or salt composition of the wash-fusion solutions. The protoplasts were fused according to the K protocol

Parameters	Fusion combination	Total protoplasts ($\times10^5$)	Survival rate (%)	Fusion products	Fusion rate ($\times10^{-3}$)	Fusion products per 10^5 protoplasts
		Number of protoplasts per dish and hormone pretreatment[a]				
2.5×10^5, no hormones	nia26 + try$^-$	12.5	8.5	17	0.26 ± 0.16	1.36
2.5×10^5, + hormones		15	18.4	122	0.53 ± 0.37	8.13
5.0×10^5, + hormones		15	18.5	222	0.80 ± 0.24	14.80
		Hormone concentration in the culture medium[b]				
Standard	nia8 + his$^-$	10	11.5	49	0.41 ± 0.15	4.90
Reduced to 1/5		10	31.1	353	1.15 ± 0.20	35.30
		Wash and incubation solution prior to fusion				
Sucrose (15 mM Ca^{2+})	nia8 + his$^-$	10	26.8	285	1.06 ± 0.25	28.50
Mannitol (15 mM Ca^{2+})	nia26 + his$^-$	10	29.7	680	2.30 ± 0.67	68
W5 salts		10	41.2	1,555	3.70 ± 0.32	155

[a] 6 mg/l NAA plus 2 mg/l BAP were added to the enzyme solution

[b] The standard concentrations are 3 mg/l NAA plus 1 mg/l BAP

Table 3. Effect on fusion efficiency of PEG composition and concentration, lenght of incubation with PEG, as well as fusion substrate. Plating efficiencies and fusion rates from more than 30 independent fusion experiments on leaf-leaf and leaf-cell suspension protoplast combinations are also summarized. Fusions were performed according to the K protocol

Parameters	Fusion combination	Total protoplasts ($\times 10^5$)	Survival rate (%)	Fusion products	Fusion rate ($\times 10^{-3}$)	Fusion products per 10^5 ppl
		Final PEG concentration[a]				
37.5 (50)		5	8.4	253	6.02±2.4	50.6
30 (40)	nia26 +his$^-$	5	20	220	2.20±0.9	44
18.7 (25)		5	18.8	111	1.20±0.8	22.2
		PEG composition (cf. Table 1)				
PEG "K"	nia8 +his$^-$	5	14.2	1,228	17.30	245.6
PEG "S"	met +his$^-$	5	30	260	1.70	52
PEG "C"	nia26 +his$^-$	7.5	16.4	78	0.63	10.4
		Time of incubation with PEG (37.5% final concentration)				
10 min.	cnx21 +cnx27	20	25.6	1,628	3.5 ±1.6	81.4
15 min.	cnx21 +cnx103	30	11	1,061	5.2 ±2.4	35.4
		Fusion substrate[b]				
On coverslip	nia8 +his$^-$	20	19	1,666	4.9 ±1.4	83.3
On plastic	cnx27 +cnx103	25	33.4	2,279	2.6 ±0.9	91.1
Plastic Petri dish	his$^-$ +try$^-$	15	45.6	1,135	1.7 ±0.6	75.6
Glass Petri dish		15	23.6	1,084	3.1 ±1.8	72.3
Plastic Petri dish	nia8 +his$^-$	20	24	904	2.2 ±1.2	45.2
Centr. tube	met$^-$ +his$^-$	25	4	282	3.5 ±3.0	11.3
		Protoplast source				
Leaf-leaf	26 experiments		22.4	–	2.8 ±2.4	–
Leaf-callus	10 experiments		19.5	–	4.9 ±2.4	–

[a] PEG concentration is given as %; the initial concentration of the PEG solution is given in brackets

[b] Greiner plastic Petri dishes were used. On glass, flat drops of protoplasts and protoplasts plus PEG are produced, in which case local densities of protoplasts and diffusion rates may be different than those found on plastic Petri dishes

2 Factors affecting fusion rates during incubation with PEG

It was of interest to establish which of the many steps involved in the PEG-agglutination process or sequence were the critical ones. Reported PEG solutions vary extensively in all their components, such as Ca^{2+} salt type and concentration, sugars, PEG molecular weight, concentration, and commercial suppliers. Kao et al. (1974) worked out a PEG solution with high PEG concentration (50%, and a final PEG-concentration during fusion of 37.5%), and no sugar. This makes agglutination and subsequent fusion very efficient. Varying single factors in Kao's and some other authors' PEG solutions revealed that: (1) empirically devised PEG solutions were remarkably balanced, and (2) it was not always possible to separate the effects of individual factors within a given PEG combination. However, several interesting facts were observed (Table 3). For example, PEG concentration was an important parameter, and molecular weight was not critical (not shown). Reducing the PEG concentration from 50 to 40% and adding an equimolar amount of mannitol, or even the simple addition of 0.1 M mannitol to the 50% PEG solution, diminished the fusion rates. Fusion of protoplasts in W5 salts allowed a reduction in PEG concentrations ($\geqq$ 15% final concentration; not shown). The period of incubation with PEG was also important, leaf-leaf protoplast combinations requiring shorter incubation periods. The Ca^{2+} source, at the high Ca^{2+} concentrations tested, was not critical. Increasing the pH of the PEG solution from pH 5.7 to pH 9 increased fusion rates (not shown) provided that fresh PEG solutions were made (or stored at –20 °C) and the pH was repeatedly adjusted before sterile filtration and use. Glass Petri dishes or glass cover slips versus plastic as support for protoplasts during fusion gave similar results, while fusion in centrifuge tubes appeared more difficult to monitor (increased frequency of damaged protoplasts, and no accurate control over the agglutination-fusion process itself).

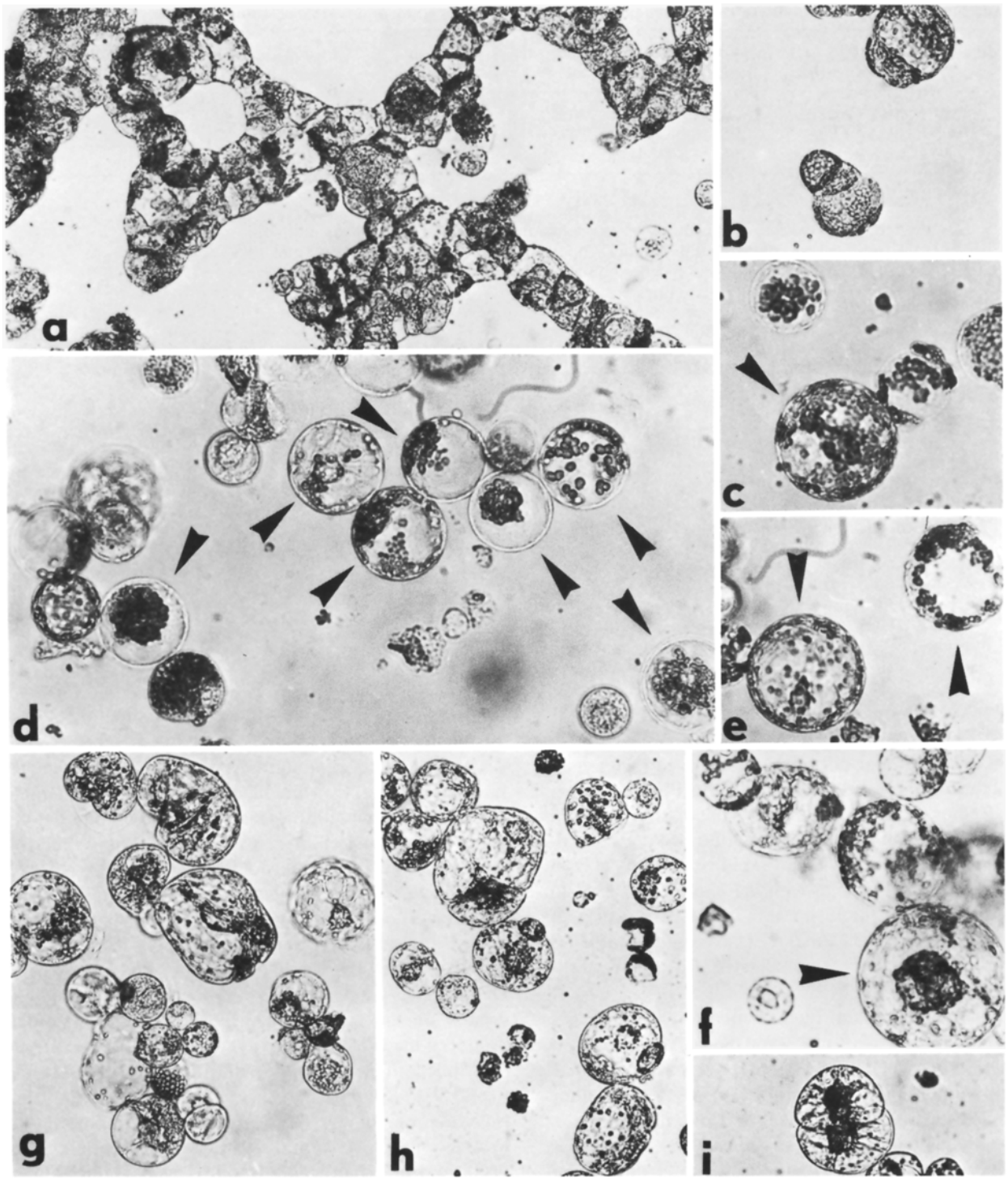

Fig. 1a–i. Sequence of events in a CMS fusion protocol between leaf protoplasts (cnx 20) and cell suspension protoplasts (A 14). Note the relatively good survival of the protoplasts after fusion. **a** typical agglutination pattern of protoplasts in PEG following sedimentation in W5; **b** rounding up of the agglutinated protoplasts during dilution of the PEG; **c, d, e** aspect of several heterokaryons (*arrows*) 1 h after fusion; a multifusion body is shown in **c**; **f** a fusion product after one day in culture; **g, h, i** first divisions in fusion products at day three in culture

Table 4. Fusion efficiency in comparative experiments with fusion protocols K, M, and CMS (see Table 1)

Procedure	Fusion combination	Total protoplasts ($\times 10^5$)	Survival rate (%)	Fusion products	Fusion rate ($\times 10^{-3}$)	Fusion products per 10^5 protoplasts
K	his$^-$ + met$^-$	2.5	20.6	410	7.9 ± 4.6	164
M	nia26 + his$^-$	25	6	6,120	40 ± 29	244.5
CMS	nia8 + try$^-$	5	16	1,280	16 ± 8.8	256

Table 5. Electric-field mediated fusion of protoplasts as a function of the DC-pulse voltage and number of pulses (p). K protocol was performed in parallel. The fusion combinations tested were met$^-$ + his$^-$ and nia26 + his$^-$. Colonies derived from batches pulsed once and two times divided more actively and were more uniform than the PEG-fused ones

Parameters	Total protoplasts ($\times 10^5$)	Survival rate (%)	Fusion products	Fusion rate ($\times 10^{-3}$)	Fusion products per 10^5 protoplasts
PEG-mediated fusion					
K	15	8.4	234	1.8 ± 0.6	15.6
Electrofusion					
250 V; 2 p	1	10.5	8	0.7	8
300 V; 2 p	3	18	115	2.1 ± 1	38.3
350 V; 1 p	1	37	127	3.4 ± 1.5	127
350 V; 2 p	1	5	10	2	10
400 V; 1 p	2	23	478	10.3 ± 3.1	239
400 V; 2 p	2	22	62	6.3 ± 4.3	31
400 V; 3 p	2	2.5	7	1.4 ± 0.6	3.5

3 Other factors tested

Unless W5 or similar salt mixtures are used (Hein et al. 1983) the desirability of removing the PEG is debatable. Removal of PEG allows the incubation of fused protoplasts at high densities during the first days of culture and, consequently, the rapid initiation of cell divisions. Alternatively, as with the K protocol, an 8- to 12-fold dilution of the fusion mix in the Petri dish with culture medium (≦ 5% final PEG concentration) does not apparently affect the survival (also see "Discussion").

As particular mutant lines or mesophyll protoplasts of certain species may not stand K or M methods, a "gentler" but still efficient procedure was worked out as a combination (and a compromise) of several other methods (the CMS procedure; see Table 1 and Fig. 1), where preincubation in W5 is followed by fusion in a 40% PEG solution at pH 9, and removal or dilution of the PEG with W5 (Table 4). Stepwise addition of salts before the removal of the fusion mix resulted in a slower deplasmolysis of the agglutinated protoplasts as compared to separate removal of PEG and wash solution. This may reduce the damaging effect of the fusion conditions.

In general, whenever W5 salts are employed, the fusion process is completed in most fusion bodies by the end of the treatments, i.e. approximately 30 to 40 min after addition of the PEG.

A few other treatments were tried and shown to have negative, positive, or neutral effects on fusion rates. Incubation with proteases for 30 to 60 min before fusion (Hartmann et al. 1976), or incubation in culture medium with 2-chloro-benzonitryl (DB) at 4 mg/l for 3, 5 or 7 days before fusion produced negative results. DB was shown to specifically block cell wall synthesis but not cell division (Meyer and Herth 1978). The fact that fusion rates fell by 10- to 20-fold after incubation with DB suggests that either some cell wall deposition occurs, or that other changes in the plasmalemma produced by delayed fusion affected the fusion process.

Embedding protoplasts after fusion into agarose (0.6–0.8%) (Shillito et al. 1983) may increase the survival rates of the fused protoplast population. An incubation of 2 to 6 h at 6°–8 °C in wash media before fusion did not significantly modify fusion rates, which means that protoplasts can either be stored or preconditioned at low temperature in various ways prior to fusion. The two different enzyme solutions used to isolate the protoplasts did not seem to affect the response of the protoplasts to fusion. Finally, no significant differences in complementation rates of the different mutant combinations tested under identical fusion conditions were observed (Tables 2 to 5).

4 A comparison between PEG- and electrofusion

Isolated protoplasts were resuspended in a mannitol solution with either 15 mM or 0.1 mM $CaCl_2 \cdot 2\,H_2O$. The former were fused according to the K protocol, while the latter were electrofused by varying both the DC pulse voltage and the number of pulses (Table 5). At the optimal 400 V and 1 pulsing, electrofusion was superior to the parallel PEG-fusion treatment. The fusion rates were below those obtained with the M protocol, but as survival rates were relatively high, the RN values were similar (compare Tables 4 and 5).

Discussion

The objective of this work was to assess various parameters affecting fusion rates, with particular emphasis on leaf-leaf protoplast fusion. Limiting the use of in vitro cultured cells as the source of protoplasts in favour of cells with balanced euploid genetic constitution, such as mesophyll protoplasts, is believed to reduce the instability of the clones and enhance the ability to regenerate plants from fusion products (Gleba and Sytnik 1984; Negrutiu et al. 1984).

Leaf protoplasts, which are usually highly synchronized in Go, are more difficult to fuse and more sensitive to chemical fusion conditions. The production of asymmetric hybrids by the fusion of protoplasts with lethally irradiated partners requires both optimized fusion conditions and a controlled quality of protoplasts. Since true auxotrophs are now available as regenerated plants possessing the euploid chromosome complement, we decided to use these to study the fusion process in some detail.

The final criteria in analysing fusion conditions were fusion rates (FR) and the relative number of fusion products per 10^5 fused protoplasts (RN) calculated after selection of stable clones exhibiting metabolic complementation on minimal media.

Measuring fusion rates at colony rather than heterokaryon level seemed the appropriate way to assess for fusion parameters and methodology in intraspecific fusion combinations because important factors such as proliferation ability and final yield of fusion products could be taken into consideration. For example, heterokaryons resulting from multifusion events are usually non-viable.

Under the conditions described, essential factors included: the physiological condition and purification degree of the protoplast population, preincubation in salt mixtures, the density of protoplasts at fusion, and PEG concentration. Variations on other factors (Tables 2, 3, 4, and 5) had little or no effects on fusion efficiency and the ease, safety, and speed of the fusion process were made preferential considerations.

The so-called M procedure (Menczel et al. 1981) gave the highest fusion rates, but – together with the K method – requires "robust" protoplasts. Fusion rates were systematically above 3 to 4% with mesophyll protoplasts. To avoid excessive multiple fusion we recommend that the density of protoplasts at fusion should be between $1\text{–}2 \times 10^6$ ml^{-1}. Whenever the W5 solution is used, the composition of the PEG solution is less important provided that the final concentration of the PEG is $\geqq 15\%$ and the Ca^{2+} concentration is around 0.1 M. Under such conditions a high Ca^{2+}/high pH treatment is optional (CMS procedure, for example).

The electrofusion procedure was also efficient, technically easy, and quicker than the PEG-fusion. Higher interelectrode distances of around 5 mm greatly simplified electrode construction, simultaneously increasing the volumes of protoplast suspensions that can be handled at much lower field strength. It must be noted that this technique needs further and more detailed studies to achieve optimal fusion conditions. Fusion is completed extremely rapidly, and leaf protoplasts seem to be more tolerant to electric damage than cytoplasmically-rich cells. The limiting factor, at present, is the apparent necessity to maintain the protoplasts at very low or without any Ca^{2+} salts before fusion which increases their fragility and requires rapid manipulation after isolation.

It becomes obvious that simple, reproducible, and more universal protocols of protoplast fusion can be worked out at present, and therefore more sophisticated studies on somatic cell genetics of higher plants can be envisaged.

Acknowledgements. This work was supported by OOA 80/85 – 8 research contract and NATO Research Grant N 738/84. Part of this work was performed at the Friedrich Miescher Institute. The authors are indebted to Stephanie Berry for helpful criticism and discussions.

References

Ashmore SE, Gould AR (1982) Protoplast fusion and the cell cycle. Plant Cell Rep 1:225–228

Bates GW, Hasenkampf CA (1985) Culture of plant somatic hybrids following electrical fusion. Theor Appl Genet 70: 227–233

Berri SF (1983) Factors affecting hybridisation between sexually incompatible species of *Petunia*. In: Potrikus I, Harms Ct, Hinnen A, Hütter R, King PJ, Shillito RD (eds) Protoplasts 1983. Birkhäuser, pp 82–84

Dirks R, Sidorov D, Negrutiu I, Jacobs M (1985) Complementation analysis of nitrate reductase-deficient mutants of *N. plumbaginifolia:* evidence for a new category of cnx mutants. Mol Gen Genet 201:339–343

Gleba YY, Sytnik KM (1984) Protoplast fusion and parasexual hybridization of higher plants. In: Shoeman R (ed) Protoplast fusion. Genetic engineering in higher plants. Springer, Berlin Heidelberg New York, pp 36–61

Glimelius K, Eriksson T, Grafe R, Müller AJ (1978) Somatic hybridization of nitrate reductase-deficient mutants of *N. tabacum* by protoplast fusion. Physiol Plant 44:273–277

Glimelius K, Wallin A, Eriksson T (1978) Concavalin A improves the PEG method for fusing plant protoplasts. Physiol Plant 44:92–96

Harms CT, Potrykus I, Widholm JM (1981) Complementation and dominant expression of amino acid analogue resistance markers in somatic hybrid clones from *D. carota* after protoplast fusion. Z Pflanzenphysiol 101:337–390

Hartmann JX, Galla JD, Emma DA, Kao KN, Gamborg OL (1976) The fusion of erythrocytes by treatment with proteolytic enzymes and PEG. Can J Genet Cytol 18: 503–512

Hauptmann R, Kumar A, Widholm JM (1983) Carrot × tobacco somatic hybrids selected by amino acid analog resistance complementation. In: Potrykus I, Harms CT, Hinnen A, Hütter R, King PJ, Shillito RD (eds) Protoplast 1983. Birkhäuser, pp 92–94

Hein T, Przewozny T, Schieder O (1983) Culture and selection of somatic hybrids using an auxotrophic cell line. Theor Appl Genet 64:119–122

Installé P, Negrutiu I, Jacobs P (1985) Protoplast-derived plants in *N. plumbaginifolia:* improving the regeneration response of wild type and mutant cultures. J Plant Physiol 119:443–454

Kao NN (1982) Plant protoplast fusion and isolation of heterokaryons. In: Wetter LR, Constabel F (eds) Plant tissue culture methods. NRCC 19876, Prairie Reg Laboratories, Saskatoon, Sask, pp 49–57

Kao KN, Michayluk MR (1974) A method for high frequency intergeneric fusion of plant protoplasts. Planta 115: 355–367

Kao KN, Constabel F, Michayluk MR, Gamborg OL (1974) Plant protoplast fusion and growth of intergeneric hybrid cells. Planta 120:215–227

Maliga P (1982) Cell culture procedures for *N. plumbaginifolia.* Plant Mol Biol Newslett 3:108–113

Marton L, Manh Dung T, Mendel RR, Maliga P (1982) Nitrate reductase-deficient lines from haploid protoplast cultures of *N. plumbaginifolia.* Mol Gen Genet 186:301–304

Menczel L, Nagy F, Kiss Z, Maliga P (1981) Streptomycin-resistant and sensitive somatic hybrids of *N. tabacum* + *N. knitiana:* correlation of resistance to *N. tabacum* plastids. Theor Appl Genet 59:191–195

Menczel L, Galiba G, Nady F, Maliga P (1982) Effect of radiation dosage on efficiency of chloroplast transfer by protoplast fusion in *Nicotiana.* Genetics 100:487–495

Meyer Y, Herth W (1978) Chemical inhibition of cell wall formation and cytokinesis, but not of nuclear division, in protoplasts of *N. tabacum.* Planta 142:253–262

Negrutiu I (1981) Improved conditions for large scale culture, mutagenesis, and selection of haploid protoplasts of *N. plumbaginifolia.* Z Pflanzenphysiol 104:431–442

Negrutiu I, Dirks R, Jacobs M (1983) Regeneration of fully nitrate reductase-deficient mutants from protoplast cultures of haploid *N. plumbaginifolia.* Theor Appl Genet 66:341–347

Negrutiu I, Jacobs M, Cattoir-Reynaerts A (1984) Progress in cellular engineering of plants: biochemical and genetic assessment of selectable markers from cultured cells. Plant Mol Biol 3:289–302

Negrutiu I, De Brouwer D, Dirks R, Jacobs M (1985) Amino acid auxotrophs from protoplast cultures of *N. plumbaginifolia.* 1. BUdR enrichment selection, plant regeneration, and general characterisation. Mol Gen Genet 199: 330–338

Power GB, Pearson EM, Heyward C, George D, Evans PK, Berry SF, Cocking EC (1976) Somatic hybridization of *Petunia hybrida* and *P. parodii.* Nature 263:500–502

Shillito RD, Paszkowsky J, Potrykus I (1983) Agarose plating and a bead type culture technique enable and stimulate development of p-derived colonies in a number of plant species. Plant Cell Rep 2:244–247

Watts JW, King JM (1984) A simple method for large scale electrofusion and culture of plant protoplasts. Biosci Rep 4:335–342

White DWR, Vasil IK (1979) Use of amino acid analog-resistant cell lines for selection of *N. sylvestris* somatic cell hybrids. Theor Appl Genet 55:107–112

Zimmermann U, Scheurich P (1981) High frequency fusion of protoplasts by electric fields. Planta 151:26–32

Zimmermann U (1982) Electric-field mediated fusion and related electrical phenomena. Biochim Biophys Acta 694: 227–277