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Conditions for Induced Fusion of Fungal Protoplasts in Polyethylene Glycol Solutions

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Abstract. Solutions containing polyethylene glycol MW 6000 (PEG) induced fusion of protoplasts of *Penicillium chrysogenum*. Balanced heterokaryons were formed by fusion of nutritionally complementing protoplasts. Heterokaryotic fusion products were obtained up to a frequency of 4% of the number of protoplasts, surviving the fusion treatment. Investigation of the conditions, necessary to achieve this high fusion frequency, showed that supplementing the PEG solution with Ca^{++} and adjustment to high pH gave the best results. Mechanisms of fusion of fungal protoplasts by PEG, calcium and alkaline pH are discussed in view of the obtained results.

Key words: Fungal protoplasts – Polyethylene glycol-protoplast fusion – Heterokaryon formation – *Penicillium chrysogenum*.

Polyethylene glycol has already been shown to have versatile activity as a fusogenic agent with different types of cells and protoplasts. Plant protoplasts (Kao and Michayluk, 1974; Wallin *et al.*, 1974), red blood cells (Ahkong *et al.*, 1975) and fungal protoplasts (Anné and Peberdy, 1975) have been fused by treatment with polyethylene glycol, but to date no real explanation exists of the mechanism by which this induced fusion occurs. To answer these questions, information on the effects of various parameters on the fusion event is required. So far, only a few reports exist on the influences of the external factors on aggregation and fusion of higher plant protoplasts (Kao and Michayluk, 1974; Kao *et al.*, 1974; Wallin *et al.*, 1974) and only Kao *et al.* (1974) gave some indications of the viability of the fused products. In this communication we describe the influence of different conditions of polyethylene glycol MW 6000 (PEG) treatments on the fusion of fungal protoplasts, estimated on the basis of balanced heterokaryons, formed by fusion of nutritionally complementing protoplasts of *Penicillium chrysogenum*, developing on a minimal medium.

Materials and Methods

Organisms and Culture Conditions. Two auxotrophic mutants of *Penicillium chrysogenum* Wis. 49-2105, *(lys-5 whi)* and *(leu met cys ylo)*, were selected after treatment with N-methyl-N'-nitro-N-nitrosoguanidine (Adelberg *et al.*, 1965) and checked for their stability over several vegetative generations. The auxotrophic mutant strains with white *(whi)* and yellow *(ylo)* conidia formed after fusion prototrophic mycelia producing white, yellow and green (wild type) conidia (Anné and Peberdy, 1975). Strains were maintained on complete medium (CM) and spores were harvested as already described (Anné *et al.*, 1974). Mycelium for protoplast formation was grown in a production medium (PM), *i.e.* a minimal medium (MM) (MacDonald *et al.*, 1963) supplemented with Difco yeast extract (2.0 g/l) and the requirements of the auxotrophs: lysine, leucine, methionine and cysteine, each 0.05 g/l. Two hundred ml of this medium in 2 l Erlenmeyer flasks were inoculated with 1×10^8 spores of each auxotroph separately and shaken for 20 hrs (250 rpm, 30° C). Regeneration of protoplasts was on solid PM or MM, made hypertonic with 0.6 M NaCl.

Protoplast Formation. Mycelium grown in PM was harvested on Whatman No. 1 filter paper and washed with an aqueous solution of 0.9% NaCl. For protoplast production, 100 mg mycelium was suspended in 1 ml of 0.55 M NaCl solution, containing 10 mg strepzyme 12,705 and 15 mg cellulase (E. Merck, Darmstadt, Germany), a crude enzyme extract from *Oxyporus* (Anné *et al.*, 1974), both containing chitinase, α- and β-glucanase. After 4 hrs gently shaking at 30° C, liberated protoplasts were separated from mycelial debris by filtration through a sintered glass filter (porosity 1) and twice washed with 0.7 M NaCl.

Fusion Treatment and Selection of Heterokaryons. Washed protoplasts ($1-5 \times 10^6$ of each auxotroph) were mixed and centrifuged (700 g, 10 min). Unless otherwise indicated, the protoplasts were resuspended in1 ml of a pre-warmed (30° C) solution of 40% (w/v) PEG (Koch-Light, Colnbrook, U.K.) in 0.01 M $CaCl_2$ and 0.05 M glycine, pH 7.5, adjusted with 0.01 M NaOH, and incubated (30° C, 10 min). After dilution of PEG with 6 ml of hypertonic MM, protoplasts were centrifuged (700 g, 5 min) and twice washed with 0.7 M NaCl. Serial

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dilutions of treated protoplasts were plated on MM and on PM. The ratio of the number of colonies on MM to the number of colonies developing on PM after 48–72 hrs was defined as

the fusion frequency or $F_f = \frac{\text{number of colonies on MM}}{\text{number of colonies on PM}}$.

Spores or mycelium, treated or untreated with PEG, and untreated protoplasts did not develop on MM, even after mixing high densities (10^7) of spores or protoplasts of each auxotroph and incubating for 14 days. Fused nutritionally complementing protoplasts regenerated on MM as heterokaryons as identified by the criteria of Pontecorvo and Sermonti (1954). Mycelium from colonies developing on MM and successively transferred onto MM always sporulated into white and yellow conidia of the parental strains. Green sectors of diploid conidia also appeared in the mixed white-yellow colonies (Anné and Peberdy, 1975). Variation in the number of heterokaryons, developing on MM, reflected the influence of fusion conditions.

Results

Factors affecting Protoplast Fusion. Cations present in the PEG solution affected protoplast fusion, but had no influence on their viability, except if 0.1 M calcium or magnesium ions were present at high pH. Ca^{++}, as $CaCl_2$, promoted the highest level of fusion and at pH 7.5 they were optimal at 0.01 M (Fig. 1), but with increasing pH the optimum molarity for fusion increased to 0.6 M $CaCl_2$ at pH 9.0. Mg^{++}, as $MgSO_4$, was stimulating to a lesser degree, but at 0.1 M it was inhibitory. Increasing concentrations of Na^+ or K^+, either as chloride or as nitrate, reduced fusion to a minimum and with 0.005 M EDTA it was almost completely inhibited. Salt concentrations at 0.0001 M had no influence and gave the same F_f as PEG solutions, buffered at pH 7.5 with 0.05 M glycine and 0.01 M NaOH, without any salt addition. Combinations of 0.01 M $CaCl_2$ with other salts at different concentrations (> 0.0001 M) resulted in a decrease of the degree of fusion.

Fusion occurred in PEG solutions at pH levels lower than pH 7.0, however, if $CaCl_2$ was present, alkaline conditions had an important influence on F_f, because the largest yield of fusion was obtained at pH 9.0 (Fig. 2). Above pH 9.0 PEG solutions were harmful causing reduced viability in the protoplasts, although they could survive in hypertonic solutions, without PEG, Ca^{++} or Mg^{++}, at pH 10.5.

The concentration of PEG was not critical within limits (Fig. 3). Protoplast aggregates composed of up to 20 protoplasts were observed after addition of PEG solution, if PEG concentrations were used at 20% (w/v) or higher; lower concentrations did not stabilize and protoplasts burst. Solutions containing 30% (w/v) PEG were optimal, they stabilized with the smallest reduction in the number of protoplasts. PEG at 40% (w/v) or higher was very hypertonic and

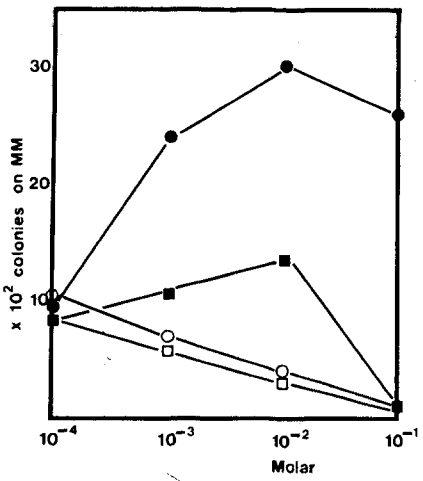

Fig. 1. Influence of different ions on the yield of balanced heterokaryons of *Penicillium chrysogenum*, developing on MM. $5.5 \cdot 10^6$ protoplasts of each auxotroph were treated (30° C, 10 min) with 1 ml of a solution of 40% (w/v) PEG, pH 7.5, adjusted with 0.05 M glycine – 0.01 M NaOH. $CaCl_2 \cdot 2\ H_2O$ (●——●); $MgSO_4 \cdot 7\ H_2O$ (■——■); $NaNO_3$ (□——□) or KCl (○——○) were added at various concentrations. The average number of protoplasts regenerating on PM was $4.3 \cdot 10^5$ protoplasts

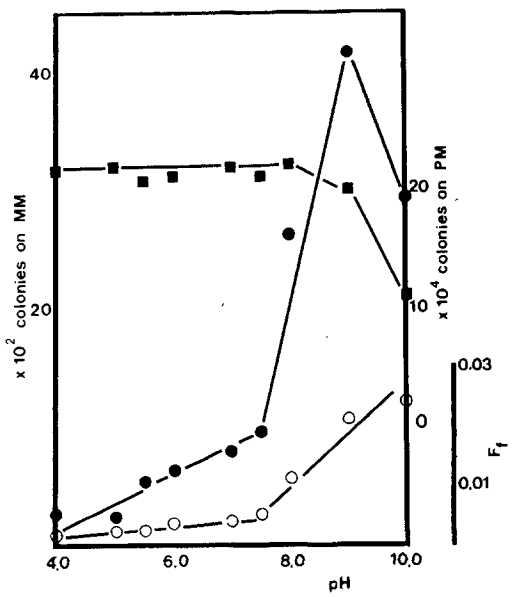

Fig. 2. Influence of pH on the fusion frequency (○——○) of *Penicillium chrysogenum* protoplasts. One ml of 40% (w/v) PEG in 0.01 M $CaCl_2 \cdot 2\ H_2O$ and 0.05 M glycine at various pH levels, adjusted with 0.01 M NaOH or HCl, was added to a mixture of $2.7 \cdot 10^6$ protoplasts of each auxotroph and incubated at 30° C for 10 min. After dilution with MM and washes with 0.7 M NaCl, fusion was expressed by the number of heterokaryons developing on MM (●——●) after 72 hrs. Total number of viable protoplasts was estimated by the number of colonies on PM (■——■)

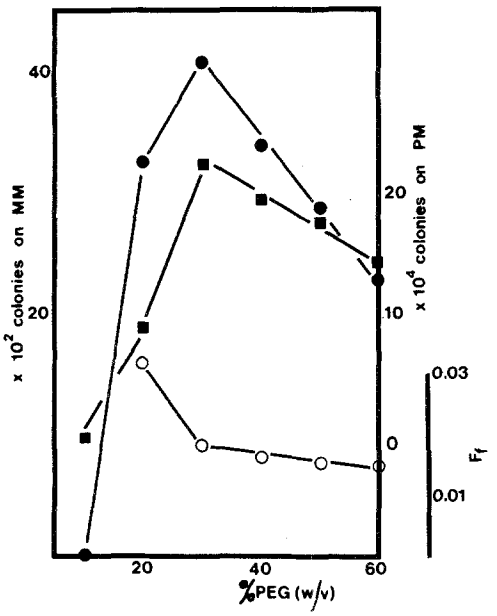

Fig. 3. Influence of concentrations of PEG on the fusion frequency (○——○) of *Penicillium chrysogenum* protoplasts. $2.0 \cdot 10^6$ protoplasts of each auxotroph were treated with PEG at different concentrations, dissolved in 0.01 M $CaCl_2 \cdot 2\ H_2O$ and 0.05 M glycine – 0.01 M NaOH, pH 7.5 (30° C, 10 min). Colonies on MM (○——○) and on PM (■——■) were counted after 72 hrs

the protoplasts shrunk, but after dilution with MM they regained their normal size. After removal of PEG by 0.7 M NaCl, the aggregates separated and larger protoplasts were observed. They were assumed to originate from fused protoplasts. If 10% (w/v) PEG solutions, osmotically balanced with 0.5 M mannitol and 0.4 M NaCl, were used, at least 0.01 M $CaCl_2$ was needed for fusion and F_f was more pH dependent (Table 1). If no PEG was added to the fusion inducing solution, fusion was only achieved if 0.05 M Ca^{++} were present at high pH, or from pH 7.5 in solutions containing 0.6 M $CaCl_2$ (Table 1).

Fusion of protoplasts took place at 4° C, but increased with increasing temperature (Fig. 4). The number of regenerating protoplasts steadily decreased and dramatically fell when incubated above 37.5° C.

The effect on aggregation and fusion occurred immediately after PEG was assumed to have spread over the surface of the protoplasts (Fig. 5). The increase of fusion during the first minutes was supposed due to the time needed for entirely coating of the protoplasts by PEG or the time needed for the reaction of all fusogenic factors in the incubation mixture with the protoplast surface. This supposition was affirmed by the fact that after longer incubation no further increase in F_f was observed.

Table 1. The influence of Ca^{++}, pH and concentration of PEG in fusion inducing solutions on the fusion frequency. 2×10^6 protoplasts of each auxotroph of *Penicillium chrysogenum* were mixed and centrifuged. Pelleted protoplasts were resuspended in the fusion inducing solution and incubated at 30° C for 10 min, except protoplast suspensions without PEG were centrifuged (50 × g, 3 min) and incubated at 37° C for 15 min. After dilution of the suspension with 7 ml hypertonic MM protoplasts were washed twice with 0.7 M NaCl and plated on hypertonic MM and PM

Fusion inducing solution[a]	Heterokaryon formation per ml on MM		Protoplasts ($\times 10^{-3}$) per ml regenerating on PM		F_f ($\times 10^2$)	
	pH 7.5	pH 9.0	pH 7.5	pH 9.0	pH 7.5	pH 9.0
30% (w/v) PEG solutions						
– without salt addition	475	5	480	45	0.10	0.011
– 0.05 M $CaCl_2$	3840	1600	380	47	1.01	3.42
– 0.05 M $CaCl_2$ 0.5 M mannitol, 0.4 M NaCl	720	2500	880	145	0.082	1.72
– 0.6 M $CaCl_2$	520	3200	360	67	0.14	4.60
10% (w/v) PEG solutions						
– 0.5 M mannitol, 0.4 M NaCl	0	0	820	325	0.0	0.0
– 0.05 M $CaCl_2$	protoplasts burst		protoplasts burst		–	–
– 0.05 M $CaCl_2$ 0.5 M mannitol, 0.4 M NaCl	5	700	625	300	0.00072	0.23
– 0.6 M $CaCl_2$	25	220	460	60	0.0055	0.36
0% (w/v) PEG solutions						
– 0.5 M mannitol, 0.4 M NaCl	0	0	760	340	0.0	0.0
– 0.05 M $CaCl_2$ 0.5 M mannitol, 0.4 M NaCl	0	65	950	640	0.0	0.001
– 0.6 M $CaCl_2$	38	0	540	10	0.006	< 0.01

[a] Fusion inducing solution was adjusted to pH 7.5 and pH 9.0 by 0.05 M glycine and 0.01 M NaOH.

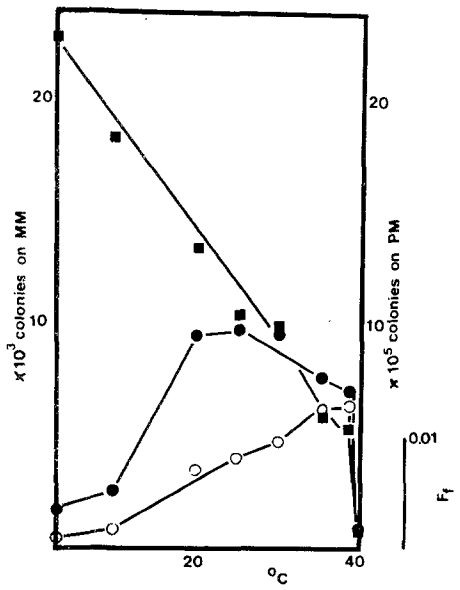

Fig. 4. Influence of temperature on the fusion frequency (○——○) of *Penicillium chrysogenum* protoplasts. One ml of 40% (w/v) PEG, pre-cooled or pre-warmed to the proper temperature, was added to $7.4 \cdot 10^6$ protoplasts of each auxotroph and incubated for 10 min at different temperatures. PEG was diluted with MM and protoplasts were washed with 0.7 M NaCl at the corresponding temperatures. Colonies on MM (●——●) and on PM (■——■) were counted after 72 hrs

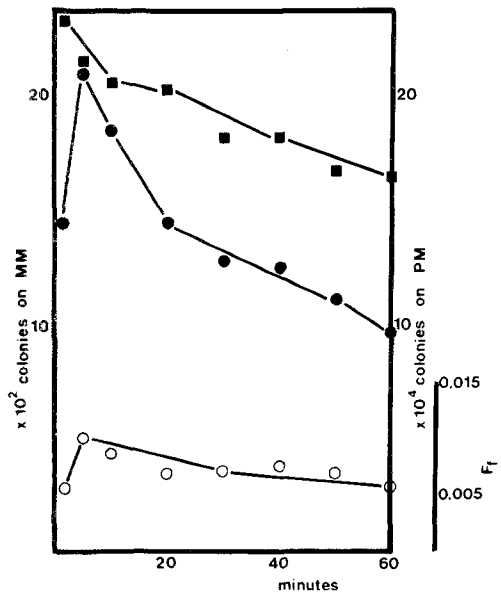

Fig. 5. Influence of exposure to PEG on the fusion frequency (○——○) of *Penicillium chrysogenum* protoplasts. The same conditions as described in Fig. 2, but 40% (w/v) PEG was used at different exposure times

Storage of protoplasts at 4° C, either in 0.7 M NaCl or in the lytic enzyme solution, improved F_f (Fig. 6), though there was a loss of viable protoplasts. In lytic

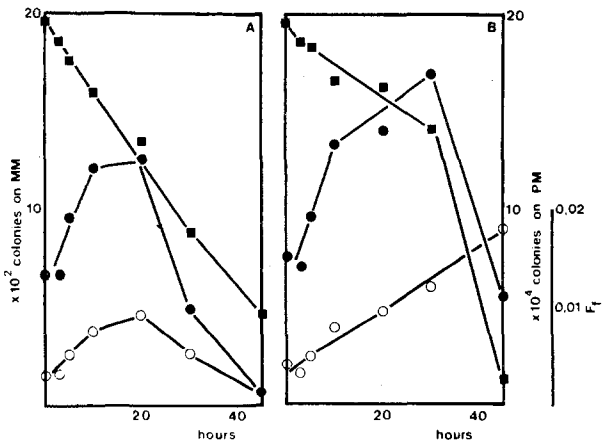

Fig. 6. Effect of storage at 4° C, (A) in 0.7 M NaCl, and (B) in lytic enzyme solution, on the fusion frequency (○——○) of *Penicillium chrysogenum* protoplasts. $2.1 \cdot 10^6$ protoplasts of each auxotroph were treated with 40% (w/v) PEG, in 0.01 M $CaCl_2 \cdot 2\ H_2O$ and 0.05 M glycine – 0.01 M NaOH, pH 7.5 (30° C, 10 min). Colonies on MM (●——●) and on PM (■——■) were counted after 72 hrs

enzyme solution F_f continued raising, but in 0.7 M NaCl fusion yield decreased faster than protoplast reduction after 20 hrs storage.

Discussion

Considering the number of fused products in relation to the parameters of PEG solution we found that high pH and Ca^{++} stimulated fusion, but concentration of PEG and time exposure to PEG were important with respect to the viability of the protoplasts. At least 20% (w/v) PEG was needed to stabilize most of the protoplasts, if no additional stabilizer was added to the PEG solution, and incubation time must be long enough for an entire contact of PEG with the protoplast surface. Too high concentration or too long incubation time resulted in a loss of viable protoplasts without affecting F_f (Figs. 3 and 5). Osmotic shock was not a factor in the fusion event as supposed by Kao and Michayluk (1974) and by Wallin *et al.* (1974), because 20% (w/v) PEG, too low to stabilize all protoplasts, gave a higher F_f compared to solutions stabilizing or overstabilizing the protoplasts.

Higher temperatures had probably a dual effect, decreasing the viscosity of the PEG solution facilitating contact with protoplasts, and making the cytoplasmic membranes more fusogenic by an increase in membrane fluidity (Ahkong *et al.*, 1973).

Enlargement of cytoplasmic volume was assumed to promote fusion during storage. Bigger protoplasts have a larger contact surface and possibly a thinner

membrane. The decrease in F_f after 20 hrs storage of protoplasts in 0.7 M NaCl, could be due to cell wall formation based on their cytoplasmic reserves, but in the presence of lytic enzyme solution cell wall formation would be inhibited. This positive effect of storage was also observed with higher plant protoplasts (Kao *et al.*, 1974).

Constabel and Kao (1974) have demonstrated the importance of Ca^{++} for increase in the fusion yield in higher plant protoplasts. They assumed that polyethylene glycol acts as a molecular bridge between adjacent membranes, either directly by hydrogen bonds, or indirectly by Ca^{++}. If no Ca^{++} are present, linkages are formed by hydrogen bonds, promoted at lower pH (Table 1). K^+ or Na^+ or Mg^{++}, in addition with Ca^{++}, diminished the effect of Ca^{++} in the fusion, though they did not have any considerably influence on the aggregation of the protoplasts as seen in the microscope and noticed by Wallin *et al.* (1974) for high concentration of K^+ or Na^+. Ca^{++} are of general interest in membrane fusion (Papahadjopoulos *et al.*, 1974); they induced fusion at high pH between erythrocytes (Toister and Loyter, 1971), higher plant protoplasts (Keller and Melchers, 1973; Binding, 1974), liverwort protoplasts (Schieder, 1974) and fungal protoplasts (Table 1) (Binding and Weber, 1974; Anné and Peberdy, 1975). If K^+, Na^+ or Mg^{++} are present, these could be preferentially bound to the membranes and decrease the amount of Ca^{++}, bound to the membranes, which is reflected in the fusion yield.

High pH may change some characters of the membranes (Keller and Melchers, 1973), and could promote Ca^{++} links with the [$^-$O-C-] ends of the PEG molecules. Increase of protoplast fusion by slow elution of PEG with high pH and Ca^{++} could be due to those effects rather than due to an increasing degree of charge disturbance (Kao *et al.*, 1974), or by the combined effects of both.

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